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CORNELL UNIVERSITY
AGRICULTURAL EXPERIMENT STATION

STUDIES ON FUNGICIDES

I. CONCEPTS AND TERMINOLOGY

H. H. WHETZEL AND S. E. A. McCALLAN

II. TESTING PROTECTIVE FUNGICIDES IN THE LABORATORY

S. E. A. McCALLAN

III. THE SOLVENT ACTION OF SPORE EXCRETIONS AND OTHER AGENCIES ON PROTECTIVE COPPER FUNGICIDES

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IMPERIAL BUREA
27 AUG. 1930
OF MYCOLOGY

ITHACA, NEW YORK
PUBLISHED BY THE UNIVERSITY
Received for publication October 7, 1929

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STUDIES ON FUNGICIDES

I. CONCEPTS AND TERMINOLOGY

H. H. Whetzel and S. E. A. McCallan

There is now under way or projected, in the Department of Plant Pathology at Cornell University, a series of investigations directed toward the solution of fundamental problems in fungicidal efficiency. It is proposed to publish the results of these investigations as units in a continuing series under the general title *Studies on Fungicides*. As introductory to the series, this first paper sets forth some concepts and terminology which appear basic and indispensable to a rational discussion of hypotheses and results to be presented in succeeding papers.

The term *fungicide* is commonly employed to designate any chemical element or compound which is toxic to fungi. It is a more restricted term than *germicide*, and is coordinate with *bactericide*, *insecticide*, *nematocide*, and the like. While commonly restricted in its application to chemical elements or compounds, it may perhaps be extended legitimately to include such physical agents as light, heat, or other forms of radiant energy. In this paper it is employed in the more commonly restricted sense. Only chemical fungicides are dealt with.

Since the toxic constituents of fungicides can act on the fungi only by diffusing into the protoplasm, it is evident that they must eventually become soluble. Many of our commonly used fungicides are immediately soluble in water. It is generally recognized, however, that certain of our standard fungicides, notably bordeaux mixture and sulfur dust, are not sufficiently soluble in pure water to exercise their fungicidal properties. A number of hypotheses have been advanced to account for the evident solution of the toxic constituents of these fungicides after they have been applied to plants. Certain constituents of the atmosphere, excretions from the plant itself, and excretions from the germinating spores, each have been held by investigators to be the dissolving agent.

Therefore, on the basis of their state in water, fungicides may be divided into two classes: (1) those soluble in water, that is, those in which the toxic principle acts directly and immediately upon the living protoplasm of the fungus; and (2) those essentially insoluble in water, that is, those in which the toxic principle can act effectively only on being liberated by some other agent than water. While all chemical elements or compounds are probably soluble in water to at least a very slight degree, the term *insoluble* is here used in the sense that the toxic principle is of itself not sufficiently soluble in water to inhibit or terminate infection by the pathogene.

Based on its function in any given case, a fungicide acts as a *protectant*, a *disinfestant*, or a *disinfectant*. It functions as a protectant, when applied to the suscept before inoculation, that is, before the inoculum has arrived in the infection court (for example, in spraying apples for scab); as a disinfestant, when applied after inoculation but before infection, that is, after arrival of the inoculum but before the pathogene has established itself in the suscept (for example, in treating seed wheat for stinking smut); as a disinfectant, only when applied after infection has taken place (for example, in spraying plants already affected with powdery mildews).

Fungicides that act to control the disease when they are applied after the pathogene has become associated with the suscept (that is, after inoculation or infection), are now commonly designated as *disinfectants*. We can speak accurately of disinfection, however, only when the fungicide acts on the pathogene after it has established pathogenic relations with the suscept. When the toxic action of the fungicide is exerted on the pathogene while it is merely in physical association with the suscept, as in the case of many seed-infesting pathogenes, it is more nearly precise to designate its action as *disinfestation*.

One has to consider, moreover, in this connection the whole problem of terminology in the matter of fungous infestation of the soil or other nonliving harborers of fungous pathogenes. The almost universally used expressions, "diseased soil," "sick soil," "infected soil," "inoculated soil," "soil inoculation," and the like, are inaccurate and unscientific in the light of our present ideas and concepts of disease phenomena. Disease is an injurious physiological process, a response on the part of the suscept to pathogenic irritation. Even if one includes (as one usually does) in the concept of disease, the chemical reactions of the dead tissues of timber to the activities of decay-inducing fungi, one certainly has no similar relationship in mind between phytopathogenic fungi and the soils in which they are present. Strictly speaking, infection is pathogenic irritation. Only living things can be infected. Soil, therefore, cannot be *infected*, dictionaries to the contrary notwithstanding; it may only be *infested* or *contaminated*. Obviously, since soil cannot be infected or diseased in any reasonable sense of these words, it cannot be "inoculated." Only living things that presumably possess the power to respond to pathogenic agents, can be inoculated. One can contaminate or infest a soil with pathogenes; one cannot inoculate it. The extensive use of "inoculate" to designate the planting of organisms on culture media is equally faulty; "planting" or "seeding" is better usage.

It follows, therefore, that one may not logically speak of "disinfecting" the soil, or of a "soil disinfectant." The terms *disinfestant* and *disinfestation* express more nearly accurately and specifically the concepts involved. The writers advocate the use of the terms *disinfect* and *disinfectant* only in

those cases in which the destruction of the pathogene results in the cessation of infection (for example, in the treatment of oat seed with formalin for control of loose smut). Here the seed is actually infected (Gage, 1927). Where destruction or elimination of the pathogene from mere physical association with the suscept or other harborers is involved, the terms *disinfest*, *disinfestant*, and *disinfestation* are to be preferred (for example, treating wheat seed for control of stinking smut, or drenching soil with a solution of formaldehyde to eliminate phytopathogenic organisms).

Water-soluble fungicides, with few exceptions, function as either *disinfestants* or *disinfectants*. They are usually effective only in cases of infestation, though sometimes they may actually disinfect, that is, destroy the pathogene after it has established pathogenic relations with the suscept. The use of lime-sulfur or potassium sulfide to destroy the powdery-mildew fungus already established on roses or other plants, is a good example of disinfection by water-soluble fungicides. There appear to be few water-soluble fungicides employed in plant-disease control which are capable of penetrating the tissues of the plant to destroy an invading pathogene without economic injury to the suscept. The action of formaldehyde in the case of loose smut of oats (Gage, 1927), and that of borax or boric acid in the disinfection of oranges infected by Penicillium (Fawcett and Lee, 1926:361), appear to be cases in point. The water-soluble fungicides most commonly employed for purposes of disinfestation in plant-disease control are formaldehyde, some mercury compounds, and certain copper compounds.

A good disinfestant is (a) toxic to the infesting pathogene, (b) practically harmless to the infested organs of the suscept at effective concentrations, (c) innocuous to the soil or other non-living harborers infested with the pathogene, (d) easy of application, and (e) economical.

Where used as a disinfectant, the fungicide must be (a) able to penetrate to the pathogene if that is within the tissues of the suscept, (b) toxic to the infecting pathogene at a concentration practically harmless to the tissues of the suscept, (c) easy of application, and (d) economically profitable in control of the disease.

Water-insoluble fungicides commonly function as *protectants*. They are usually effective only when applied to the plant before it has become inoculated with the spores of the pathogene. Bordeaux mixture is the classical protectant. Its protective function was clearly perceived by its discoverer, Millardet (Crandall, 1909:205). Next to bordeaux mixture, sulfur in its elemental state, either in suspension (dry mix) or as dust, is perhaps the most widely used protectant. Lime-sulfur, a water-soluble fungicide, at first thought might be assumed to function directly as a protectant. A moment's reflection will at once discover the fallacy in this assumption. It is chiefly after the lime-sulfur has dried and has been

converted by the action of the carbon dioxide of the air into other compounds and free sulfur, that its protective function is exercised (Haywood, 1907). The elemental sulfur precipitated from the lime-sulfur chiefly functions as the protectant.

It has long been known, though not generally appreciated, that certain essentially water-insoluble arsenical compounds — for example, arsenate of lead and arsenate of lime — exhibit marked protective action against the apple-scab pathogene, *Venturia inaequalis* (Butler and Doran, 1928). The writers and T. C. Loh (Whetzel, McCallan, and Loh, 1929) have recently reported that calcium arsenate exhibits marked specificity against *Alternaria panax*, a pathogene but slightly susceptible to the toxic action of copper or sulfur protectants.

That insoluble fungicides may sometimes act as disinfestants, or even as disinfectants, is not to be denied. The elimination of powdery mildews by applications of sulfur dust to infected plants may be looked upon as a case of disinfection by an insoluble fungicide. Such cases are, however, few and exceptional.

A good protectant is (a) practically insoluble in pure water, (b) highly adhesive to the surface of the suscept organs to be protected, (c) active only in the presence of some solvent, (d) essentially harmless to the suscept, (e) miscible with insecticides if desired, the efficiency of neither being impaired nor the suscept endangered, (f) easy of application, and (g) economical.

It is evident, then, that water-soluble fungicides act chiefly as disinfestants or disinfectants, while water-insoluble fungicides function, with few exceptions, as protectants.

In the foregoing presentation, the writers offer what they hope may appeal to plant pathologists as a much-needed clarification of the chaotic state of the terminology of our current concepts of fungicides and fungicidal phenomena. It is believed that we have now reached a point in the development of the science of plant pathology where a revision in our terminology, consistent with the present state of our concepts in this field, will conduce to more nearly accurate and more logical thinking. It may be argued that general usage justifies the application of terms in a sense contrary to their strict meaning. This position can hardly be maintained, however, in the face of the current confusion in our terminology of this phase of plant pathology. Neither is such a position tenable on the ground of want of words adequate to convey the specific concepts involved. Even where such words are not already available, the language offers abundant material for acceptable coinage. The concepts and terms presented in this paper have long been employed by the senior author in his college course on the principles of plant-disease control.

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STUDIES ON FUNGICIDES
II. TESTING PROTECTIVE FUNGICIDES IN THE LABORATORY

S. E. A. McCallan

The laboratory testing of fungicides to determine their toxic properties has gained ground rapidly during recent years. The growing interest in this matter is doubtless due, in part at least, to the ever-increasing number of proprietary compounds on the market, a condition which makes field testing all but prohibitive in time and labor. In view of these facts, a short discussion at this time relative to the general scope of fungicidal tests in vitro, and the presentation of a method developed in the Laboratory of Plant Pathology at Cornell University and long in use there, may be serviceable to other workers. Such a discussion is presented in the following pages, and will serve as an introduction to the technic employed in certain studies on fungicidal action which constitute the third paper of this series.

Experience here has demonstrated, over a period of years, that the toxicity of a protective fungicide can be most readily and effectively determined in the laboratory. When the difficulties involved in measuring the other requirements of a good protectant (page 6) under the unnatural conditions of the laboratory or even of the greenhouse are considered, it is probable that testing under natural field conditions will give, for the present at least, the most reliable results for such factors as adhesiveness, toxicity to the plant, and the like.

A study of the literature on the nature of fungicidal action shows that, in general, one of three hypotheses usually has been advanced to explain the liberation of the active fungicidal principle from the insoluble protectant. These hypotheses are: (1) that this is accomplished by means of atmospheric agencies, especially rain, dew, and the carbon dioxide in the air; (2) that it is accomplished by the suscept, especially by the excretions from the organs to be protected; and (3) that it is accomplished through a solvent action exerted by the fungus spores themselves upon the protectant.

The literature relative to the first hypothesis is much in conflict, and experiments by the author, which are still in progress, show that, at least for certain common protectants, the rôle played by atmospheric agencies is almost negligible. Convincing evidence to substantiate the hypothesis of suscept excretions is lacking in the literature. The data on the hypothesis of spore excretions are very limited and are somewhat in

Author's acknowledgments. The writer desires to acknowledge his indebtedness to all those workers of the Laboratory of Plant Pathology at Cornell University, both past and present, who have been instrumental in the development of the method of testing fungicides here described. Especial thanks are due to Professor H. H. Whetzel, of the Department of Plant Pathology, for the valuable advice rendered by him in the progress of these studies.

conflict. However, the author's experiments strongly support the third hypothesis. The evidence for this is presented in the next paper of this series.

It is evident that if fungicidal action involves essentially only a relationship of spore and protectant, the laboratory testing of fungicides, confined also to this relationship, may be expected to be both reliable and practicable. The advantages of laboratory tests lie in the saving of time, expense, and labor, and in the more definite control of environmental conditions. A set of tests may be made in a month or two which under field conditions would require several years. Thus, from many possibilities a few probabilities may be selected for actual field testing.

It must be emphasized that in the testing of fungicides the determination of their toxicity is of primary importance. The toxicity having been determined in the laboratory, the investigator is then in a position to proceed to the field for tests of adherence and injury to the plant. Spraying or dusting experiments on a large scale in the field must serve as the final test in the evaluation of any fungicide.

In the preparation and selection of fungicides, laboratory testing is playing an increasingly important rôle. Not only in many phytopathological research laboratories does this constitute a significant part of the program, but also in commercial laboratories.

The practicability, advantages, limitations, and uses of laboratory tests of fungicides have been outlined. What, then, is necessary in a reliable method of testing the toxicity of protective fungicides in vitro? First of all, it must duplicate, in so far as possible, actual field conditions. Some methods depart from this requirement to a large degree. Wallace, Blodgett, and Hesler (1911:167) state:

> It is true that many experiments have been conducted to determine the effect of certain fungicidal solutions on spore germination. In most such cases, however, the spores were placed directly in a solution or mixture of the fungicide. The results, therefore, must be considered as of scientific interest rather than as serving to indicate what these same substances will do after being sprayed and dried on the plant. The latter is the condition in which fungicides, with very few possible exceptions, must prevent infection.

Other considerations call for a method that is accurate and quick, with a simple technic, and capable of wide application.

DEVELOPMENT OF THE METHOD

In 1910 Reddick and Wallace published a brief note in *Science*, entitled *On a Laboratory Method of Determining the Fungicidal Value of a Spray Mixture or Solution.* This was the first published record of this method, the fundamental principle of which was developed by Dr. Reddick in connection with some class work during the winter of 1908 (Wallace, Blodgett, and Hesler, 1911). A few months later, Wallace, Blodgett, and Hesler (1911) devoted considerable space to a discussion of the method.

In explanation they say, in part (page 167 of reference cited): "We must, therefore, expect that progress in the discovery of new fungicides or beneficial modifications of old ones will be slow until some method is adopted whereby the *fungicidal properties* of various substances can be *studied in the laboratory.*" Since that time the method has been more or less continuously employed in this laboratory by various workers who have introduced modifications and improvements at different periods. During the past three years the author has had occasion to use the method very extensively. Naturally, some new problems have presented themselves, and, as obstacles have arisen, attempts more or less successful have been made to overcome them. As now developed, it is believed that this method of testing approaches actual field conditions more nearly than does any other described in the literature.

TECHNIC

The technic of the method as now in use is as follows:

The fungicide to be tested is sprayed or dusted on standard 3x1-inch clean glass slides which are conveniently placed on a tray. Every effort must be made to get an even and well-distributed layer of the fungicide. (The matter of application is presented in more detail later.) With such dusts as copper-lime, which should be dusted only on wet foliage, the slides must first be sprayed with distilled water until they are coated with fine, uniform droplets. The dust is then immediately applied. Liquid sprays are left exposed to the air and allowed to become completely dry. This will usually take several hours. The slides are then ready to be placed in the moist chambers.

Moist chambers are used in order to keep, without evaporation, the drops of water in which the spores are placed to germinate. These chambers have been found more suitable than petri dishes, since four slides may be placed under exactly the same conditions. Also, the air relations are better. In order that the chambers may be sealed with water, they are inverted for use. A satisfactory size of moist chamber is 7 by 20 centimeters. A piece of clean filter paper is placed in the bottom of the chamber. This tends to hold the moisture and to prevent the slide-holding racks from displacement, and provides an excellent background for purposes of observation. The slides must be held above the moist filter paper or the water. To accomplish this, glass slide racks are used, one for each chamber. These may be easily constructed of glass tubing bent to form a U. The length of the rack should be sufficient to easily hold the four slides parallel to one another, and yet allow room for the chamber cover to be placed in position (figure 1).

A suspension of the spores to be tested is prepared by carefully washing them from the substrate into the particular kind of water desired. If

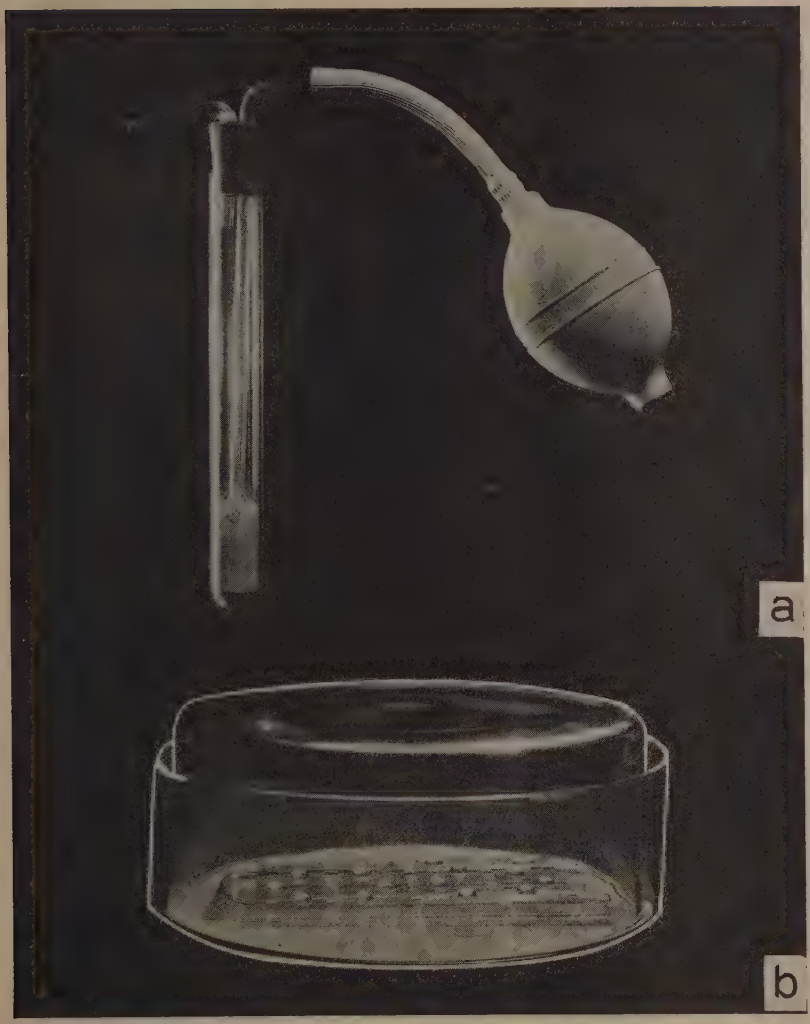

FIGURE 1. TEST-TUBE DUSTER (a) AND MOIST CHAMBER (b)

The moist chamber is equipped with filter paper, slide rack, and slides, for a spore-germination test. The spores are suspended in the drops of water on the slides. The chamber has not yet been sealed with water

necessary, the spore suspension is filtered through cheesecloth or cotton to remove bunched spores and pieces of mycelium or of substrate. The spore concentration of the suspension is regulated by dilution until, as ascertained by examination of representative drops under the microscope, the desired concentration has been attained. Test tubes have been found most suitable for holding the suspension, since with these it may be readily agitated and kept uniform. By means of a pipette that will deliver about twenty drops to a cubic centimeter, the spore suspension is pipetted as drops on the slides. The optimum number of drops per slide is four. A greater number tend to run together, and a less number results in a decrease in manual efficiency and loss of replications. There will thus be sixteen drops to a chamber. To insure that the drops contain as representative a number of spores as possible, it is well to draw a fresh pipette-full of spore suspension for at least every two slides. Only one fungicide should be placed in a chamber; experience has taught the necessity of this. The moist-chamber cover is put in place, and water is poured around it into the bottom. This seals the chamber and preserves an atmosphere approaching saturation. Drops may be kept in this manner for a number of days before complete evaporation results. A moist chamber completely equipped and with the drops of spore suspension present, but just prior to the final act of sealing with water, is illustrated in figure 1, b. Controls are of course maintained, drops of spore suspension being placed on clean blank slides. One moist chamber containing four slides will usually constitute a satisfactory control. Thus the spores are allowed to germinate undisturbed at their optimum temperature. When the time has elapsed (determined for the particular fungus) in which a maximum number of spores in the control have germinated, producing vigorous germ tubes, the results may be taken by recording the percentage of germination and the general vigor of the germ tubes.

IMPORTANT FACTORS

In conducting these tests there are a number of factors that must be taken into consideration. For convenience these may be discussed in the order in which they appear, and may be classified under the following general headings: clean glassware, standardization of the fungus and of the conditions for spore germination, and application of the fungicide. Disregard of any of these factors gives unsatisfactory results.

CLEAN GLASSWARE

It is very essential that all the glassware be clean. The free use of chromic-acid cleaning mixture is necessary, for the slides must be chemically clean. The cleaned slides should be stored and protected from dust. At the end of each experiment, the moist chambers and the slide

racks must be thoroughly washed with water. The use of dirty or used moist chambers markedly affects the results of experiments.

The necessity for sterile conditions has not been felt in the use of this method. Interest is only in germinating the spores, and if ordinary precautions are taken to prevent undue contamination it has been the writer's experience that satisfactory results can be obtained. It is, of course, entirely possible that with spores of certain fungi sterile conditions will be necessary.

Other than the fungicide, no foreign substance should be placed in the chamber. Clark (1899), Duggar (1901), and Melhus and Durrell (1919) have noted that the presence of such foreign substances as carbon, paraffin, and vaseline has stimulated spore germination. Glue and balsam used to cement certain types of slide racks have greatly influenced spore germination in this laboratory. The glass-tube type of rack now used carries no foreign material and can readily be washed.

STANDARDIZATION OF THE FUNGUS AND OF THE CONDITIONS FOR SPORE GERMINATION

These factors are of paramount importance, though unfortunately often disregarded. It is very essential, if one plans to work with any fungus on a large scale, that it should be "standardized"; that is, the optimum factors for spore germination should be determined, and all conditions controlled, in so far as possible. Unless the control spores germinate consistently, no significant comparisons of toxicity can be made. This is because spores of lowered vitality are greatly influenced by any adverse condition, such, for example, as treatment with a toxic agent. The result of this is a low germination out of all proportion to that of the control when compared with treated and control spores of greater viability.

Source of spores

Ideally, perhaps, the spores should be obtained from nature, growing on their natural substrates. This is not always possible, however, especially during the winter months, when the tests are most likely to be made. Furthermore, the writer has found that spores obtained directly from nature generally vary more in their germinating ability than do those grown on artificial media. The spores from culture are thus more satisfactory to work with. Where the fungus is to be grown in culture, the choice of a suitable medium must be made. A suitable medium is one on which the fungus will produce an abundance of spores, and spores that will give maximum germination.

The maintenance of a high percentage of germination by spores from artificial culture following isolation from nature may present a problem. It was found, for example, that conidia of *Sclerotinia americana* obtained

from rotting cherries gave a germination of about 94 per cent. As winter approached, the fungus was isolated and grown on potato-dextrose agar. At first the germination was very poor, conidia from the second transfer giving only 10 per cent of germination. Conidia from the third transfer germinated better, but it was not until the fourth transfer that the spores again gave their maximum germination, this time about 98 per cent — slightly higher than in nature. It has been found also, with certain fungi, that after many generations on artificial media the spores produced tend to lose their viability. An effort was therefore made to keep the number of generations to a minimum by always transferring from the oldest culture available. The fungus *Sclerotinia americana* has been grown continuously in culture for thirty months, new plantings being made about every week to obtain spores for germination tests, and yet only the fourteenth generation has been reached and the fungus still maintains its high germination ability of 98 per cent.

Age of culture

Although the age of the culture is not a definite indication of the age of the spores produced thereon, there is nevertheless a more or less well-defined correlation. The common practice has been to specify the age of the spores by the age of the culture at the time when the spores are taken for the test. In comparative tests the age of the spores should be known, and preferably they should be of the same age. Doran (1922: 316), in an interesting contribution to our knowledge of the factors influencing spore germination, has concisely stated, "Until the spore has attained a certain age or degree of maturity, it cannot germinate," and also (page 318 of same reference), "For all spores there is a maximum time limit, variable with the environmental conditions, beyond which the spore cannot germinate. The spore may be alive and too old to germinate or it may be dead." This definite time limit within which the spore will germinate should be borne in mind. Even within this limit there is a fairly well-defined period of optimum germination; and it is a point of major importance that as the age for optimum germination is passed the spore becomes more susceptible to the toxic effects of fungicides.

This relationship of spore age to toxic sensitivity in the case of 300-mesh dusting sulfur and *Sclerotinia americana* is well shown in figure 2, which has been compiled from the results of two experiments. The rapid drop in the sulfur-germination curves points to the extreme importance of the age factor and the necessity of knowing these relations when making fungicidal tests. A similar effect of copper-lime dust on the germination of *Macrosporium sarcinaeforme* spores is shown in table 1. Doubtless this factor applies also under field conditions, but this point cannot easily be proved.

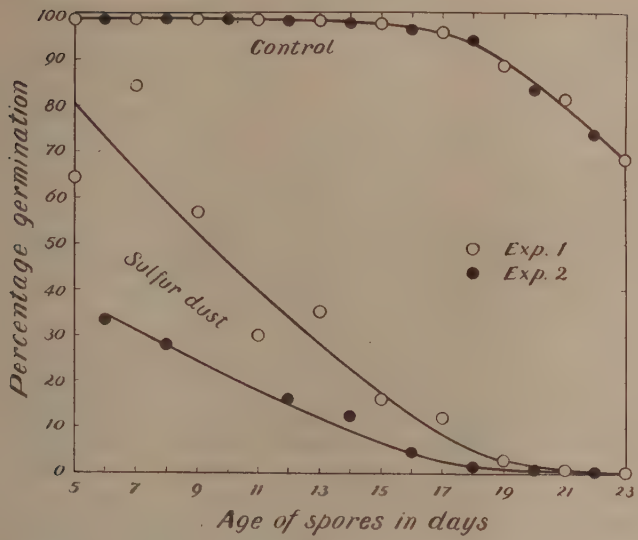

FIGURE 2. TOXICITY OF SULFUR DUST AS RELATED TO THE AGE OF THE SPORE: PERCENTAGE GERMINATION OF CONIDIA OF SCLEROTINIA AMERICANA

TABLE 1. RELATION OF SPORE AGE AND TOXICITY OF COPPER-LIME DUST, TO PERCENTAGE GERMINATION OF MACROSPORIUM SARCINAEFORME*

Age of spores, in days	Per cent germination	
	Control	Copper-lime
16	98.1	9.7
24	97.3	2.9
35	97.1	1.7
54	95.0	1.0
75	95.6	1.1
95	93.9	0.6

* Data from results obtained by Dr. J. G. Horsfall, formerly of this laboratory.

Kind of water

Among the external factors affecting germination, it is most logical to consider first the type of water best suited for the germination of the spores. In general, for this purpose there is distilled, tap, or rain water, the last including lake, stream, and spring waters. The last-named type

of water will of necessity vary greatly. Although meteoric waters — that is, rain or dew — will be present under natural conditions, it is felt that they are not ideal for tests because of their great variation in composition. Distilled water would seem to be the most suitable, provided the spores will germinate well in it. Doran (1922) found that the distilled water which he used lacked aeration, and unless it was artificially aerated it reduced germination. Lack of aeration has not proved a serious obstacle in the writer's studies. All the spores that he tested required precipitated moisture for germination. In some cases, spores germinate equally well merely in a moist atmosphere (Doran, 1922). Certain types of spores are placed in suspension in water only with the greatest difficulty, and as yet no method has been devised to satisfactorily overcome this.

Concentration of spore suspension and oxygen relations

All workers have found that the presence of oxygen is essential for normal spore germination (Uppal, 1926). In all probability there is never an excess of air dissolved in the water, as far as the spore requirements are concerned. Hence the size and shape of the drops of water on the slides must be considered. It has generally been found that the flatter the drop, the better will be the germination. Also, the drops should all be of the same size in order that the oxygen relations and the possible staling effects may be constant. Finally, unless the drops are of the same size, surface and vapor-tension phenomena will result in the larger drops increasing in size at the expense of the smaller ones.

Doran (1922:332) noted "the injurious effect of the presence of fungous spores on the germination of the spores of other fungi." He attributed this to a competition for oxygen. He states: "When there is lack of sufficient oxygen for all, only those spores which require the least will germinate." Obviously, only one kind of spores should be present in the drop.

Because of the oxygen limitations and the staling effect (Wallace, Blodgett, and Hesler, 1911:173), there is a limit also to the number of spores that will germinate in a given drop of water. Hence there is an optimum spore concentration. As a general rule it may be said that the fewer the spores, the higher is the percentage of germination. However, if the number of spores is very much reduced, the probable error for germinations in a single drop will become unnecessarily high. Therefore, in making up spore suspensions in water the object should be to obtain as high a concentration of spores as is possible without unduly reducing the germination.

The relations of spore concentrations to germination for *Botrytis allii* are shown in table 2. In this, as in all succeeding tables, the number of

spores is based on a low-power microscopic field of 1575 microns diameter. Here, as can be seen, it is not advisable to have a spore concentration greater than 50 spores per field.

TABLE 2. SPORE-CONCENTRATION AND GERMINATION RELATIONS FOR BOTRYTIS ALLII IN WATER

(80 per cent sterile lake water, 20 per cent tap water, 21° C.)

Number of spores	Per cent germination
20– 50	92.5
51– 75	88.4
76–100	85.4
101–125	82.5
126–150	72.3
151–200	67.7
201–300	48.5

The relations of spore concentrations to the kind of water, for the conidia of *Botrytis allii* and *Cephalothecium roseum*, respectively, are shown in tables 3 and 4. For *Botrytis allii* lake water is seen to be the most suit-

TABLE 3. SPORE-CONCENTRATION AND WATER RELATIONS FOR BOTRYTIS ALLII

(Percentage germination at 21° C.)

Number of spores	Per cent germination		
	In lake water	In tap water	In distilled water
15– 25	97.0	92.2	94.5
26– 50	95.5	72.2	61.7
51– 75	93.5	71.5	43.4
76–100	91.2	.....	.....
101–150	84.1	.....	4.3

TABLE 4. SPORE-CONCENTRATION AND WATER RELATIONS FOR CEPHALOTHECIUM ROSEUM

(Percentage germination at 21° C.)

Number of spores	Per cent germination		
	In rain water	In tap water	In distilled water
20– 50	50.3	30.7	62.7
51– 75	36.6	24.6	60.0
76–100	12.7	23.2	56.1
101–150	10.7	22.8	34.2
151–200	8.2	19.3	26.3
201–300	.....	.....	18.0
301–400	.....	.....	9.2

able, and for *Cephalothecium roseum* distilled water. With each in its best-suited water, germination rapidly decreases when a spore concentration of 50 is exceeded. In no case within the experience of the author has the optimum spore concentration exceeded approximately 50 spores in a microscopic field of 1575 microns diameter. Indications are that the germination will be slightly increased as the number of spores is reduced from 50; the difference, however, is not significant. As a general rule, a spore concentration of 15 to 50 has been found the most satisfactory.

Temperature

The temperature relations for spore germination are probably better known than is any other factor. The optimum temperature for the germination of the spores of any fungus should be ascertained before laboratory tests are attempted. In table 5 is shown the effect of temperature and spore concentration upon spore germination for the conidia of *Sclerotinia americana*, and in table 6 the effect of temperature and kind of water for conidia of *Glomerella cingulata*.

TABLE 5. Temperature and Spore-Concentration Relations for Conidia of Sclerotinia americana

(Fifth transfer, 8 days old, in distilled water. Percentage germination)

Temperature (C.)	Spore concentration			
	20–50	51–100	101–200	201–400
	Per cent germination			
12.0°	42.2	11.8	0.0	0.0
15.5°	59.0	29.4	0.0	0.0
17.5°	93.9	50.5	11.1	0.4
21.0°	98.1	77.8	36.7	0.8
23.0°	99.0	90.5	57.1	7.1
25.5°	99.0	91.8	55.0	9.5
27.0°	96.5	90.0	31.7	1.0
30.0°	80.5	20.0	5.1	0.0

TABLE 6. Temperature and Water Relations for Conidia of Glomerella cingulata

(Percentage germination)

Temperature (C.)	Per cent germination		
	In rain water	In tap water	In distilled water
12°	28.3	32.2	23.4
15°	38.5	31.4	51.5
18°	61.6	55.5	67.2
21°	67.3	76.3	69.0
24°	64.1	84.7	75.6

These tables show, as do the preceding ones, that, where two factors are varied, as the optimum of the one is reached differences due to the other tend to be minimized. Thus, for example, in the determination of the optimum temperature it might be well to vary another factor also in order that the temperature differences may be shown the more clearly.

The optimum temperature for spore production may not necessarily be the same as that for spore germination. An abundance of spores is essential, and it may therefore be necessary to maintain the cultures at a temperature for spore production which is different from that for optimum spore germination. Limited tests here seem to indicate this for conidia of *Venturia inaequalis*.

Light

None of the fungi tested exhibited any preferential light requirements. This appears to agree with the results of most other investigators (Doran, 1922).

Time

Since the time limits for the germination of spores vary greatly, it will be necessary to determine this period for each fungus. If the test is discontinued too soon, not all the spores capable of germinating will have done so; if the test is continued too long, the spores may have germinated to such an extent as to form an indistinguishable tangle, not to speak of the possibility of the drop's evaporating. Of course, in nature the optimum conditions for germination may be present for such a short time that but few spores may ever germinate successfully. In spite of this, since fungicidal tests are chiefly comparative in character, it is advisable to allow sufficient time for optimum germination. Doran (1922) has compiled from the literature an interesting list of the lengths of time required for representative fungus spores to germinate.

APPLICATION OF THE FUNGICIDE

It is very essential that care be exercised in the application of the fungicide to the slides. For applying liquid fungicides a DeVilbiss atomizer, "Atlas Spray No. 29," has been found very satisfactory and will deliver the fine, uniform, mist-like spray desired. The dry fungicides, or dusts, are applied by means of test-tube dusters, one of which is illustrated in figure 1, a (page 11). These were devised by Dr. F. M. Blodgett, of this laboratory, and have long been in use here. They are now widely employed in other laboratories as well (Lee and Martin, 1927:316). They are very simple in construction, being made of a heavy-walled six-inch test tube, a two-hole rubber stopper, two pieces of bent glass tubing, and a rubber atomizer bulb. Glass tubing of 5 millimeters outside diameter

and 3 millimeters bore is very suitable. Two pieces are bent in a gradual L curve. The shorter delivery piece is placed to project just below the rubber stopper, while the longer piece, to which the atomizer bulb is attached, is just immersed in the dust. For best results the test tube should contain about an inch of dust. A separate test-tube duster must be used for each dust. A thorough washing is of course essential before the tube is used for another dust. If the dusters are to be used at frequent intervals, the dust may safely be left in the tube.

If consistent results are to be obtained, it is imperative that an even layer of spray or dust be applied to the slides. If too much spray is applied, it will tend to coalesce in large drops. A fine, even mist is the most satisfactory. It is more difficult to get an even layer of dust. Large particles may cause trouble, though oftentimes these may be blown off without disturbing the finer dust. The tendency is to apply too much rather than too little dust. In nature the average leaf seldom receives a heavy coating of dust or spray.

The general impression is that the greater quantity of fungicide used, the greater is the fungicidal efficiency. Accordingly some limited tests were carried out to study this point. Light, medium, and heavy applications of Niagara 300-mesh sulfur, Niagara Kolodust, and lime-sulfur 1:40, were made. The spores used were conidia of *Sclerotinia americana*. The results are shown in table 7.

TABLE 7. Results of Tests to Determine the Relation between Quantity and Efficiency of a Fungicide

(Percentage germination of *Sclerotinia americana* conidia at 24° C. in distilled water)

Fungicide	Application	Per cent germination		
		Experiment 1*	Experiment 2*	Experiment 3*
Niagara 300-mesh sulfur	Light	49.4 B	92.4 B+	76.2 B+
	Medium	35.8 C	78.3 C	57.8 B–
	Heavy	36.3 C	77.2 C	37.5 C
Niagara Kolodust	Light	24.6 C	70.0 C–	10.0 D
	Medium	1.0 D	17.3 D	1.0 D
	Heavy	0.3 D	7.5 D	0.0
Lime-sulfur 1:40	Light	0.0	1.8 D	.....
	Medium	0.0	Trace D	.....
	Heavy	0.0	Trace D	.....
Control	.....	94.7 A	98.6 A	96.6 A
Age of spores, in days	.....	15	13	17

* See page 21 for interpretation of letters.

Similar results were obtained with the uredospores of *Uromyces hybridii*. These are given in table 8.

TABLE 8. RELATION OF QUANTITY AND EFFICIENCY OF SULFUR DUST TO GERMINATION OF UROMYCES HYBRIDII UREDOSPORES*

(Distilled water at 21° C.)

Fungicide	Application	Per cent germination
Niagara 300-mesh sulfur	Light	85.1
	Heavy	92.8
Niagara Kolodust	Light	63.3
	Heavy	58.2
Control		92.6

* Data from results obtained by Dr. J. G. Horsfall, formerly of this laboratory.

The results of these experiments, though exceedingly limited, would seem to indicate that, provided a certain minimum of sulfur is applied, increasing the amount beyond this will not appreciably increase the fungicidal efficiency.

RECORDING RESULTS

As has already been mentioned, the test should not be concluded and the results taken until all viable spores have germinated. There are three common ways of expressing the toxicity of a fungicide: one can record the percentage of germination, can record the character of the germ tubes, or can attempt to grow the treated spores as was done by Lee and Martin (1927) and by Young and Cooper (1917). There are objections to all three. With this method of testing fungicides under asterile conditions, the last-named practice is impossible. Extended measurements of germ tubes involve much time and labor and are of doubtful value since the tests are only relative. The method first employed by the writer was merely to record the percentage of germinated spores. This was later supplemented by observations on the length and the character of the germ tubes. The germ tubes are provisionally classed into four types, A, B, C, and D, of which A is the largest and most vigorous, normally corresponding to those in the check, and D is the poorest. In general, D type includes spores that have just germinated and all up to those that have produced a germ tube approximately 25μ long; C type includes those with germ tubes 25 to 100μ long; B type, those with germ tubes 100 to 200μ long; and A type, those with germ tubes 200μ in length and longer. In addition, the signs + and − may be employed in each class. From the observations of Kightlinger (1925), it is probable that spores of D type, and probably also of C type, would be incapable of producing infection. The spores are not classified individually, but as a whole into one type for each microscopic field.

The drops of spore suspensions cannot all be made exactly the same size, nor will they encounter exactly the same conditions. Therefore, in order to avoid magnifying small differences, the general rule has been never to count more than one microscopic field of spores in a single drop. Occasionally, because of a limited number of spores it may seem advisable to count more than one field to a drop, but in this case the same number of fields should be counted in each drop. Assuming 25 spores in one field, and one moist chamber to a test, 400 spores will have been counted for one test. If possible, it is more convenient to use the low-power objective in observing the spores. In this case a cover slip will not be required. For convenience in counting, the field may be divided into quadrants. To accomplish this, a cover slip on which thin lines are drawn in ink at right angles to and bisecting each other, is placed on the ocular diaphragm of the microscope. To facilitate and expedite the counting, two Veeder Hand Tally Counters are used, one to record germinated and the other ungerminated spores. Absolute germination percentages should be recorded, and not relative germinations where all controls are raised to 100 and the other readings elevated in proportion as has been done by some workers. This latter method ignores the disproportionate effect of treatment on spores of lower viability, discussed earlier, and masks the variation between tests so that true comparisons cannot be made.

It is impossible to completely control all the conditions surrounding the laboratory testing of fungicides, especially in view of the variability and the sensitiveness of most spores (Duggar, 1901). An average of 400 or 500 spores to a test will tend to give, in the germination results, a somewhat high probable error. A repetition of tests is therefore very necessary. This may be accomplished by performing duplicate or triplicate moist-chamber tests, or by repeating the experiment. One should not expect to duplicate the figures of a former experiment. In table 9

TABLE 9. Results of Sulfur Tests on Conidia of Venturia inaequalis

(Percentage germination at 21° C. in distilled water)

Treatment	Experiment 1	Experiment 2	Experiment 3	Average
Niagara 300-mesh sulfur	18.3	39.5	44.6	34.1
Banks' colloidal sulfur diluted with Bentonite: 0.3 per cent sulfur	18.4	34.5	49.2	34.0
Banks' colloidal sulfur diluted with Bentonite: 1.0 per cent sulfur	13.0	27.5	49.2	29.9
Banks' colloidal sulfur diluted with Bentonite: 10 per cent sulfur	....	9.6	24.0	16.8
Niagara Kolodust (colloidal sulfur 10 per cent; dusting sulfur 90 per cent)	9.2	2.8	23.8	11.9
Bentonite	39.2	44.6	64.0	49.3
Control	47.2	50.6	69.5	55.8

are listed the results of three experiments on the effect of sulfur dust on the conidia of *Venturia inaequalis*. This gives an indication of the variation obtained between experiments performed at different times.

SUMMARY

1. The practicability, advantages, and limitations of laboratory tests for fungicides are discussed. It is believed that such tests are reliable and significant.

2. A method for testing developed at the Laboratory of Plant Pathology at Cornell University is described. Briefly, the technic is as follows: Glass slides are sprayed or dusted with the fungicide to be tested, and are then placed in inverted moist chambers. The spores of the fungus are suspended in water, and drops of this suspension are placed on the sprayed or dusted slides and on the check slides. The moist chamber is sealed with water, giving a highly saturated atmosphere in which the spores are left to germinate at the desired temperature. After a given time, counts are taken to determine the percentage and the type of germination.

3. The necessity for standardization of the conditions for germination and the factors relative thereto, such as the source and age of spores, type of water, spore-suspension concentration, oxygen relations, temperature, light, and time, is discussed.

4. Details concerning methods of applying the fungicides and the recording of data are presented.

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STUDIES ON FUNGICIDES

III. THE SOLVENT ACTION OF SPORE EXCRETIONS AND OTHER AGENCIES ON PROTECTIVE COPPER FUNGICIDES[1]

S. E. A. McCallan

In the first paper of this series it was pointed out that protective fungicides, or protectants, are in general insoluble in water. Kahlenberg and True (1896) have shown that the toxicity of copper salts is due to the ions in solution. Therefore, in the case of copper protectants it may generally be assumed that copper ions effect the toxic action and hence account for the protective efficiency of the fungicide. The question then arises as to the manner in which the copper is rendered soluble, since presumably it cannot exercise its toxic properties while in the insoluble state. It is thus evident that, though the copper protectant must be essentially insoluble in water, yet in the presence of pathogenic spores it must become soluble to a sufficient extent to kill the fungus, or at least to prevent infection of the suscept.

This paper sets forth the results of some studies on the manner in which the soluble copper is liberated from the nominally insoluble copper compounds. No attempt has been made to study the actual toxic action, or the manner in which the copper kills.

Most investigations relating to the nature of the fungicidal action, or, more specifically, the liberation of the copper from copper protectants, have dealt with bordeaux mixture. This is to be expected, as bordeaux mixture is our oldest and most important copper protectant. The studies here described have likewise dealt with this protectant, and also with copper-lime dust.

The chemical and the physical nature of the compound or compounds resulting from the reaction of copper sulfate and calcium hydroxide to form bordeaux mixture, are of great complexity and variability. Despite the extensive investigations on this subject, there is yet much to be clarified. The first workers, Millardet and Gayon (1885, 1887 b), and other early workers, such as Chester (1890), considered the reaction simple, holding the active fungicidal constituent formed to be cupric hydroxide. Sostegni (1890), Fairchild (1894), and Swingle (1896) state that the copper is present as a basic sulfate in addition to the hydrate. More recent workers — Pickering (1907, 1909), Bedford and Pickering (1910), and

[1] Also presented to the Faculty of the Graduate School of Cornell University, April, 1929, as a major thesis in partial fulfillment of the requirements for the degree of doctor of philosophy.

AUTHOR'S ACKNOWLEDGMENTS. The writer desires to acknowledge his indebtedness to Professor H. H. Whetzel, of the Department of Plant Pathology, Cornell University, at whose suggestion these studies were undertaken, for the stimulating encouragement and helpful advice which he constantly afforded. Sincere thanks are due also to Professors F. M. Blodgett and H. E. Thomas, of the same department, for suggestions and criticisms.

Butler (1914, 1923) — as the result of their investigations are inclined to the view that various basic sulfates of copper are formed, in addition, of course, to the calcium compounds. The actual basic copper sulfates formed are said to depend largely on the ratio of copper sulfate to lime-water or milk of lime. However, Bell and Taber (1907), the only investigators to apply the phase rule to this study, claim that the composition is constant provided a fixed temperature is maintained, and that (page 636 of reference cited) the toxic action of the copper must "be attributed either to the solid copper hydroxide (and it is difficult to see how this could act unless in solution), or else to the very dilute copper solution."

A most important feature is the colloidal nature of bordeaux mixture (Swingle, 1896, and Lutman, 1912, 1916), for it is upon the peculiar blue precipitation membranes that the protective value of this compound rests.

A good discussion of the nature of copper-lime dust, an approximate equivalent of bordeaux mixture, is given by Boyd (1926). Holland and Gilligan (1928) have presented a general classification of copper fungicides.

HISTORICAL REVIEW

A review of the very extensive literature on the liberation of the copper from bordeaux mixture reveals that, in the main, three hypotheses have been advanced to explain the freeing of the copper from the colloidal membranes. These hypotheses are stated by Barker and Gimingham (1911:76) thus:

1. That copper is brought into solution by atmospheric agencies — more especially by the action of the carbon dioxide of the air, *i. e.* a purely chemical explanation.
2. That the leaves, on to which the mixture is sprayed, exert a solvent action on the compounds, *i. e.* an action of the host plant.
3. That the fungus itself is responsible for the production of the soluble copper by which it is finally poisoned, *i. e.* an action of the fungus.

It is not the intention to present here an exhaustive review of the literature. In general, only those articles containing original experimental evidence in support of these respective hypotheses are summarized. More recent articles are discussed in somewhat more detail.

ATMOSPHERIC AGENCIES

The hypothesis assuming that atmospheric agencies free the copper from bordeaux mixture, has, without doubt, received the most attention, and perhaps at the present time is the most generally accepted. This hypothesis had its origin in the very development of bordeaux mixture itself, for in 1887 Millardet and Gayon (1887 a:161) stated (translation of original): "In the first place, it happens that water from rain and dew, in consequence of its carbonic acid and carbonate of ammonia, dissolves a sufficient amount of copper hydroxide which is on the leaves,

either to completely prevent the germination of conidia of Peronospora in this water, or at least so that it cannot take place in a normal manner." Again, to quote these investigators (1887 b:700, translation): "We have proved that the copper hydroxide contained in a drop of the mixture is soluble in rain and dew only when the lime which in the actual mixture is found in enormous excess is completely neutralized, especially by the carbon dioxide of the atmosphere." Millardet and Gayon based this conclusion on an experiment in which they prepared five types of bordeaux mixture, ranging from copper sulfate very slightly in excess, to that containing lime eight times in excess, of neutralization. These mixtures were exposed to the rains. The time of the first appearance of copper in the solution varied from five to eighteen days. Clark (1902:43), Crandall (1909:225), Bedford and Pickering (1910:72), Barker and Gimingham (1911:94), and Lutman (1916:17) all criticize this conclusion, and point out that there is no delay in the fungicidal action of the copper but that it is effective from the time of application. Clark (1902:43), in fact, thinks this should be "sufficient to have set aside the hypothesis which accounted for the solution of the copper of the bordeaux mixture by atmospheric conditions."

Sostegni (1890), experimenting with bordeaux mixture, passed carbonic-acid gas through the copper hydrate and basic sulfates and succeeded in bringing copper into solution. He therefore assumes that the CO_2 of the atmosphere has a solvent action on the precipitated copper compound. These conclusions are accepted by Lodeman (1892).

Fairchild (1894) is inclined to the view that the copper compounds are slowly dissolved by the rains and dews. He also was able to obtain soluble copper on the addition of CO_2.

Though performing no experiments himself, Swingle (1896) suggests various possibilities. He mentions the possible rôle of atmospheric agencies, but says (pages 19-20 of reference cited): "It seems scarcely possible that the minute quantities of CO_2 contained in rain water or dew alone could dissolve enough copper to poison any but the most delicate plants. Probably, as claimed by Gayon, the copper hydroxide present is slightly soluble in pure water."

Bain (1902) seems to have shown conclusively that the injurious action of soluble copper on peach leaves, resulting from spraying with bordeaux mixture, copper hydroxide, and dicupric hydroxide ($Cu_3O_2(OH)_2$), is most pronounced when the plants have been exposed to the rains and dews. This injury was found to be further accentuated in the presence of sunlight.

Ruhland (1903), studying the effect of plant excretions on the solution of basic copper, found that rain water containing CO_2 and other substances from the air could dissolve very small amounts of copper. How-

ever, he did not consider that this amount of copper exercised any fungicidal action, except possibly as a secondary effect in the case of very sensitive fungi, though he conceded that it probably was a factor in spray injury.

Schander (1904) does not believe that atmospheric agencies are ordinarily very effective. He placed various bordeaux mixtures in crystallizing dishes, one lot being exposed to the sun and the other kept in the shade. Both lots were periodically moistened with rain water. Soluble copper appeared in neutral bordeaux after 23 days, but in alkaline bordeaux not until 42 to 56 days. Butler (1914:142), however, questions the trustworthiness of Schander's test for soluble copper.

Crandall (1909) performed extensive field tests with apple trees sprayed with bordeaux mixture. The trees were either exposed to the rain and dew, or wetted with cistern water, carbonated water, or distilled water, and the drip was collected and analyzed for soluble copper and alkalinity. The freshly made bordeaux mixture contained no free copper. The drip waters did not give a neutral reaction until late in the summer, but soluble copper was present from the first and continued as long as any bordeaux was present, thus attesting to the slow rate of solubility of the copper from bordeaux mixture. Rain water and carbonated water were equally effective in bringing copper into solution. Cistern and distilled water were only about half as effective. The amount of soluble copper present was from a trace to 1 part in 11,000 parts of water. Excess lime diminished the extent of the solubility of the copper, but did not entirely prevent it. Limited laboratory experiments, however, gave contradictory results. Bordeaux mixture in crystallizing dishes, either dry or intermittently wet with cistern water, gave no soluble copper at the end of 65 days. Nor did sprayed trees, also either dry or frequently moistened with cistern water, show any copper in the wash waters at the end of 54 days. Crandall (1909:226) concludes "that meteoric waters possess solvent powers that do not belong to the water artificially applied." He assumes that the CO_2 is the chief dissolving agent, but that it is probably supplemented by other agencies, possibly ammonium compounds, nitrates, and nitrites.

Pickering (1907, 1909), and Bedford and Pickering (1910), undertook numerous laboratory experiments in an effort to demonstrate the solvent action of the CO_2 of the atmosphere. Carbon dioxide was passed through suspensions of the various basic sulfates of copper, the length of time required and the amount of copper brought into solution being noted. Copper from the Woburn bordeaux ($4CuO, SO_3$) was found to be immediately soluble, while in the case of ordinary bordeaux ($10CuO, SO_3, 3CaO$) there was a considerable delay. Pickering states that this delay is due to the presence of excess lime. The CO_2 carbonates the lime most

readily, though it acts to some extent on the basic sulfate of copper, producing ordinary copper sulfate. The excess lime still present, however, reconverts this soluble copper sulfate to the basic sulfate. This cycle continues, finally resulting in only a part of the total copper being brought into solution, and then not until nearly all of the excess lime has been carbonated.

In nature there is no appreciable delay in the fungicidal action of bordeaux mixture. Pickering explains this apparent contradiction by stating that in the laboratory the basic sulfates were constantly wet and any soluble copper found would be immediately precipitated by the lime-water. In nature the bordeaux mixture dries, and the particles of basic sulfate and of lime become separated from each other. Hence, when the former is attacked by the CO_2 there may be no lime particles sufficiently near to decompose the copper sulfate formed. In view of the fact that fungicidal protection is usually exerted only when the spores are on a wet surface, this explanation seems hardly sufficient. Pickering considers this theory established by a simple laboratory experiment, in which filter paper is moistened with potassium-ferrocyanide solution and dried. When a drop of bordeaux mixture is placed on the paper, there is no reaction as long as it is wet. However, immediately on drying, the lime particles no longer have access to the particles of basic sulfate, and soluble copper appears as is shown by the formation of red copper ferrocyanide.

Gimingham (1911) also passed CO_2 through suspensions of the various basic sulfates similar to those used by Pickering (1907, 1909) and by Bedford and Pickering (1910). In all cases the resulting soluble copper was immediately reprecipitated on the removal of the CO_2. This led Gimingham to believe that the soluble copper was probably in the form of a carbonate dissolved in the CO_2 solution. When the action of the CO_2 was prolonged, the soluble copper did not easily reprecipitate. The action of ordinary air brought little or no copper into solution. Gimingham concluded that only in the presence of a large excess of CO_2, such as could hardly occur out-of-doors, is an appreciable amount of copper brought into solution, and that "it appears impossible to assign the fungicidal action to copper sulphate liberated by *atmospheric* carbon dioxide" (page 75 of reference cited).

Barker and Gimingham (1911:86) state: "The fungicidal action of the bordeaux compound was evidently not in any way dependent upon the presence of carbon dioxide." This conclusion is based on an experiment in which conidia of *Nectria ditissima* and *Sclerotinia fructigena* were sown on a fresh precipitate of no-excess-lime bordeaux ($10CuO, SO_3$). In the absence of CO_2, there was very poor germination and any feeble growth was soon halted. The response of the controls, exposed to the air, was the same.

Pickering attempts in a later paper (1912) to refute the criticisms of Gimingham (1911) and of Barker and Gimingham (1911), and presents new evidence. Air was passed through suspensions of the various basic sulfates and copper carbonate in which bright iron rods were placed. According to Pickering, the loss in weight of the iron rods should give the amount of soluble copper abstracted from the suspension, and this is a much more delicate test than that by the potassium ferrocyanide. Soluble copper was detected, but in the case of bordeaux mixture there was none until all the free lime had been carbonated. The respective amounts of copper removed after three hours were: in the case of $4CuO, SO_3$ – 7.3 per cent; $10CuO, SO_3$ – 4.2 per cent; $10CuO, SO_3, 3CaO$ – 2.3 per cent; and $2CuO, CO_2$ – 1.0 per cent. In another experiment, air containing various percentages of CO_2 was passed through a suspension of $10CuO, SO_3$ for three hours. The relative amounts of copper brought into solution were: 0 per cent CO_2 – 109; 0.03 per cent CO_2 - 100; 10 per cent CO_2 – 58; and CO_2 only – 834. The fact that, with the exception of an atmosphere of CO_2 only, the solvent action is correspondingly greater as the amount of CO_2 in the air is reduced, reflects seriously on the possible rôle of atmospheric CO_2 in bringing copper into solution. Pickering (1912: 274), however, maintains that the fungicide would be "bathed in an atmosphere of the carbon dioxide evolved from the leaves themselves."

These conclusions of Pickering (1912) were further criticized by Barker and Gimingham (1914 b). These workers point out that the amount of CO_2 present in the atmosphere would retard rather than promote the solution of copper, according to Pickering's own results. Also, they note that the CO_2 from the leaves is rapidly dispersed. This assertion is based on the results of an experiment in which a large strawberry plant was covered with Woburn bordeaux paste ($10CuO, SO_3$) and "placed in a bowl of water under a bell-jar in order to keep the atmosphere saturated. Under these conditions the action of any CO_2 respired by the leaves would presumably be at a maximum" (page 221 of reference cited). After three days no copper could be detected in the condensed water on the leaves. The potassium-ferrocyanide test was employed.

Groth (1910), studying bordeaux injury on peaches, was able to render the copper soluble with distilled water and tap water as well as with rain water. He found also that the lower surface of the leaf was apparently more sensitive to injury than the upper. Peach leaves have no stomata in the upper epidermis. In the laboratory, injury occurred only when there was a covering of water on the leaves, a high relative humidity, and some cloudy weather. Groth therefore offers the theory (page 19 of reference cited): "An excess of CO_2 evolved in the shade, passes into the water standing on the leaf during wet weather. CO_2 is dissolved, and the carbonated water thus formed dissolves some of the copper."

Butler (1914) carefully reviews the literature relative to the action of CO_2 on bordeaux mixture. He appears, however, to have performed but few experiments himself. In one case he found that carbonated bordeaux mixture containing 0.5 per cent of cupric sulfate was not toxic to spores of *Phytophthora infestans* or of *Plasmopara viticola* in tap water, but that when distilled water was used, regardless of the ratio of cupric to calcic oxide, the mixtures were toxic at 0.0625 per cent. This difference, he explains, is due to the fact that tap water contains calcic bicarbonate in solution, and " calcic bicarbonate is a precipitant of soluble copper in the electro-positive condition " (page 139 of reference cited); hence, because of this, the tap water is unable to dissolve the copper in bordeaux mixture. Butler therefore concludes, from this experiment, that bordeaux mixtures after carbonization are slightly soluble in pure water, and that they will dissolve readily in water containing CO_2 in solution — the latter presumably a conclusion drawn from the literature. Finally, he holds that these mixtures are soluble also in water containing ammonic salts in solution. No experimental proof is presented for this last conclusion.

Pantanelli (1920), from his investigations, is inclined to the view that copper sprays are efficient for long periods, as the hydroxide is slowly acted upon by the CO_2 and rendered less basic and more soluble.

Cerasoli (1926) advances the hypothesis that many of the components of bordeaux mixture, such as the basic sulfates of copper, are hydrolyzed by water to form gelatinous copper hydrate. The copper hydrate dissociates very slightly, giving copper and hydroxyl ions. Exposed on the leaf, the carbonic acid derived from the CO_2 of the atmosphere and of plant respiration furnishes hydrogen ions. These hydrogen ions combine with the hydroxyl ions of the base to form water which is practically undissociated. The product of the copper and hydroxyl ions then becomes too small, and the equilibrium is destroyed by a further dissociation of the copper hydrates. This production of copper and hydroxyl ions will continue as long as there remains any undecomposed copper hydrate. In such a manner the sprayed plant parts are coated with an infinitesimal amount of soluble copper, which is sufficient to inhibit the germination of fungus spores. Cerasoli offers no experimental evidence.

Schmidt (1924) reviews the literature briefly, and states that of some six theories only two can now be considered as probable; namely, that of Millardet on the action of atmospheric CO_2, and that of the solvent action of the spores as advanced by Ruhland (1903) and others. With these two hypotheses in mind, Schmidt undertook various experiments. Excess-lime bordeaux mixture was made, potassium ferrocyanide was added, and leaves of apple, pear, and grape were sprayed. Only after a lapse of time did the red color appear to show the presence of soluble copper. Similar results were obtained on filter paper. A little potassium

ferrocyanide added to copper hydroxide in distilled water, and the whole placed in a dish covered with paraffin, showed no color after a month, whereas a similar mixture in an open dish showed the red color after one day.

Botrytis spores were placed by Schmidt in distilled water in an open dish and allowed to germinate for 48 hours. A control without spores was exposed for 4 hours. To each there was then added a little copper hydroxide, and after 30 minutes a test for soluble copper was made with potassium ferrocyanide. The test was positive and of equal intensity for both.

In another experiment Schmidt allowed distilled water to stand on copper hydroxide for four weeks, and then filtered. Botrytis spores were placed in the filtrate, and also on the precipitate, to which fresh distilled water had been added. These were each divided into two series; to one a little sugar was added, and to the other none. The results after three days were good germination in the precipitate, both with and without sugar, about one-third germination in the filtrate with sugar, and no germination in the filtrate without sugar. From this Schmidt concludes that the four-weeks-old filtrate was more toxic than the precipitate suspension in fresh water, and that this difference was due to the time of standing exposed to the atmosphere.

Schmidt also prepared four sets of tubes, in each of which was placed a suspension of copper hydroxide in distilled water. Trichothecium spores were sown in two of the sets, and the ends of the tubes of one set were sealed with paraffin. One of the two remaining sets was likewise sealed. After standing thus for 48 hours, potassium ferrocyanide was added to all and the color reaction was observed in 5 hours. The two sealed tubes gave a very slight reaction for soluble copper, while the unsealed tubes gave a very decided reaction, thus showing the presence of soluble copper. There was no apparent difference in the tubes with or without spores. This test was repeated with bordeaux mixture and spores of Botrytis and Aspergillus. The results were more marked this time, in that there was no reaction at all in the sealed tubes.

A similar type of experiment was set up by Schmidt, in which washed copper hydroxide was placed in watch glasses in distilled water. Half the glasses were placed in a desiccator over potassium hydroxide. The other half were left exposed to the air. To half of each of these sets, Botrytis spores were added and allowed to germinate for 48 hours, when potassium ferrocyanide was added to all. After another period of 48 hours there was only a very slight positive test for copper in the glasses in the desiccator, but after only 5 hours there was a very positive test in the exposed dishes. Schmidt states that the spores in all the dishes gave about 50 per cent germination. This appears very inconsistent with the fact that he found such marked differences in the amounts of soluble copper in the two sets.

In still another experiment, designed more or less to duplicate natural conditions, Schmidt stretched collodion membranes over one side of glass rings. Copper hydroxide or bordeaux mixture in agar was placed on the underside of the membranes. The rings were divided into two lots, and to one of the lots sugar was added. On the outside of the membranes, spores of Botrytis, Monilia, or Fusicladium were sown, along with the fungicide. With the copper hydroxide, all the spores germinated freely while in contact with the copper-hydroxide particles, either with or without sugar. With the bordeaux mixture, however, with or without sugar, there was restricted germination, though any germination or subsequent growth was slightly better where sugar was added. This experiment was further modified and set up in six series. Series 1 and 2 consisted of watch glasses, series 1 containing 2 per cent of copper hydroxide, and series 2 containing 2 per cent of bordeaux mixture, in distilled water. A trace of sugar was added to each. Series 3 and 4 were glass slides, sprayed, in the case of series 3 with 2 per cent of copper hydroxide, and in the case of series 4 with 2 per cent of bordeaux mixture. These were allowed to dry in the air. Series 5 and 6 consisted of glass rings over which the collodion membrane was stretched. In series 5 a little copper hydroxide was rubbed over the surface of the membrane, and on the inside a paste of 2 per cent of copper hydroxide with a trace of sugar was added. Series 6 was treated in the same way except that bordeaux mixture was used instead of copper hydroxide. Spores of Botrytis were sown on all six, the whole series was placed in moist chambers, and the spores were allowed to germinate for six days. The results were as follows: series 1,100 per cent germination: series 2, 20 per cent; series 3, 100 per cent; series 4, 2 per cent; series 5, 100 per cent; series 6, 0 per cent; control, 100 per cent.

In a final test, Schmidt found that Botrytis and Aspergillus germinated only very poorly in limewater. However, limewater apparently did not hinder the germination of Trichothecium spores.

Schmidt concludes from his studies that there is no exosmosis of substances from the spores which promotes the solution of bordeaux mixture or of copper hydroxide. He concludes also that bordeaux mixture is more toxic than copper hydroxide, and that the fungicidal action of bordeaux mixture is due to the combined influence of the copper brought into solution by the carbonic acid of the atmosphere, and the repressive alkalinity of the mixture.

Schmidt did not demonstrate the repressive alkalinity of bordeaux mixture toward all his spores. Apparently, he assumes that the copper of bordeaux mixture is in the form of copper hydroxide. The copper hydroxide employed was very evidently soluble in distilled water to a very appreciable extent, since Schmidt used the potassium-ferrocyanide test, which is not very delicate. In the two last-described experiments a possible solvent

action by the spores toward the bordeaux mixture is entirely possible. Schmidt's failure, in his first experiment, to obtain soluble copper from the sprayed bordeaux, is not in entire accord with his general conclusion, and also points to a delay in fungicidal action, which has been definitely established not to be the case.

In summarizing the literature on the action of atmospheric agencies, it should be emphasized that a great many of the experiments were performed with freshly made bordeaux mixture or other copper suspensions before they had been dried, as is the case when applied to plants. This evidence, therefore, can have no conclusive bearing on the liberation of copper from fungicides after they have been dried on the plant. Nor should the action of excessive quantities of CO_2 be taken as a criterion for judging the probable action of the very small quantities present in the atmosphere. There seems to be convincing evidence that meteoric waters possess a solvent action not exhibited by distilled water. Whether this difference is due solely to the CO_2 content has not been demonstrated. Under field conditions, with the plants exposed to the rain and dew, copper appears to be brought but slowly into solution from " insoluble " copper protectants.

SUSCEPT EXCRETIONS

The hypothesis that excretions from the sprayed plant (the suscept) exert a solvent action on the insoluble copper protectants, has received but little attention. From the mass of literature on injury and the physiological relations of bordeaux mixture and the suscept, there may be gathered a little direct experimental evidence relative to the solvent action of suscept excretions. The author makes no claim to having thoroughly reviewed this literature.

Swingle (1896:20) states: " It is possible that the drops of rain or dew standing for some time on the leaves or other parts of plants may absorb from superficial cells sugar or other substances that may serve to increase the solubility of the copper." Some observations of previous writers which led Swingle to suggest this hypothesis are presented.

Clark (1902) applied a spray of excess-lime bordeaux mixture to a peach tree. Following this, the tree was sprayed several times with " pure " water, to give the effect of dew. On the following day, water collected from the leaves showed the presence of a slight amount of soluble copper. Branches containing some of these leaves were placed in distilled water, and after some hours soluble copper was detected. Clark believes that there is an exosmosis of some of the contents of the cell sap, which, coming in contact with the bordeaux adhering to the leaf surface, cause more or less copper to become soluble. One is hardly justified, however, in drawing such a general conclusion from a limited experiment.

Bain (1902), studying bordeaux injury to peaches, performed a few

experiments relative to the solvent excretion from leaves. He found that the gum secretion from peach leaves did not increase the injury. By the use of blue litmus solution no acid excretion could be demonstrated from the leaves. Peach leaves, paraffined glass slides, and plain glass slides were sprayed with copper hydroxide, and were then placed in the sunlight and the water allowed to evaporate. Following this, the surfaces were kept continuously wet with distilled water for several hours. This water from the treated surfaces was then filtered, and tests were made with cells of Spirogyra to detect soluble copper. The percentages of cells killed were, respectively, 88, 24, and 51, from which Bain (1902:93) concluded that "there is *something* escaping from the peach leaf which has a solvent action on copper hydroxide." Bain did not, however, give the percentages of cells killed in his controls, and in a preliminary experiment he found that the filtrate from a stock suspension of copper hydroxide was equally as toxic as that from the sprayed peach leaves. He assumes, in common with the earlier workers, that the copper of bordeaux mixture exists in the form of copper hydroxide.

Schander (1904), also studing bordeaux injury, discovered that the leaves of Fuchsia and Oenothera excrete acids which apparently can dissolve small amounts of copper hydroxide. In the case of *Phaseolus multiflorus*, the leaf excretions reacted alkaline and Schander was unable to demonstrate a solvent action.

Ruhland (1903), as a result of his investigations, was unable to substantiate the hypothesis of suscept excretions. Studying those plants especially susceptible to bordeaux injury, he found that various organic salts, especially malic acid and potassium, were exuded through healthy uninjured leaves and fruits. However, none of these substances was capable of dissolving any more than insignificant amounts of basic copper. In fact, similar traces of copper were dissolved by pure water containing carbon dioxide, and by water from the surfaces of corked-over organs, such as twigs, which did not exude any such substances. Ruhland was unable to demonstrate the presence of exuded sugar. He therefore concludes that the traces of soluble copper on the leaves are brought into solution by rain water containing carbon dioxide and other substances from the air.

Crandall (1909) states that he was unable to obtain any evidence that the solution of copper occurs through the agency of leaf secretions. No direct experiments are mentioned.

Bedford and Pickering (1910:109–112) planned experiments in which leaves of apple, pear, and damson plums were placed in suspensions of copper carbonate and copper oxide. The leaves were later removed and tests were made for the presence of soluble copper. Solutions in which leaves, apparently uninjured, had been placed, showed the same small quantity (0.00001 per cent to 0.00002 per cent) of soluble copper as did

the controls. However, when the leaves were injured or ground up, as much as 0.009 per cent of copper was found in solution. These investigators conclude that there is no evidence that leaf excretions ordinarily are agencies in the solution of copper. The results do not appear to warrant such an unmodified conclusion. Unfortunately the leaves were detached in all cases.

Barker and Gimingham (1911) placed healthy shoots of gooseberry, pear, currant, and apple, while still attached to the trees, in jars containing a small quantity of bordeaux mixture in distilled water, and afterward, on analysis of the water, could detect no soluble copper. However, when no-excess-lime bordeaux was used, very appreciable quantities of soluble copper were detected. The controls showed none. Parallel tests in the laboratory with detached leaves gave the same results. Leaf scorching was correlated with the presence of soluble copper. Later experiments by these investigators (1914 a, 1914 b, 1914 c), on bordeaux injury to apple leaves, showed that, in the case of leaves from summer foliage, scorching by bordeaux resulted only at spots where an injury, naturally or purposely made, allowed contact between the copper compound and the inner thin-walled cells. With leaves from late autumn foliage, the scorching was more general. The injury from no-excess-lime bordeaux was much more pronounced in all cases. When uninjured apple leaves were immersed directly in copper-sulfate solutions, very little general injury resulted. Barker and Gimingham concluded that injured foliage possesses a solvent action not exhibited by uninjured foliage. Presumably this is because the interior cells have readily permeable walls unlike the heavily cuticularized outside walls of the epidermis.

The experimental evidence on the hypothesis of solvent action by suscept excretions is meager. There seems to be no satisfactory proof that uninjured leaves exercise any solvent action. Injured leaves, on the other hand, appear in all cases to exert some solvent action. However, it must be borne in mind that fungicidal toxicity is definitely exhibited in vitro, so that even though the excretions from injured leaves may be contributory to the solution of copper, they cannot be the sole agents.

SPORE EXCRETIONS

Since the present investigations have been primarily directed toward a study of the hypothesis that spore excretions exert the necessary solvent action on the compounds, a fairly comprehensive review of the literature is presented.

Swingle (1896) was apparently the first to suggest the action of the fungus spores themselves. He states (page 21 of reference cited):

> Finally, it is possible that substances secreted or excreted by cells of parasitic fungi may effect the solution of a small amount of copper. The ferments and cements secreted by the

hypha tips of many parasitic fungi, especially when they come into contact with a solid body and in case the solid particle contains copper, might dissolve a considerable amount just about the delicate growing tip.

If, as is quite possible, the copper is quickly absorbed by the fungous cells, the solvent could then take up another portion, which in turn would be absorbed by the fungus, and so on. In this manner poisoning might ensue, even though the copper were only very slightly soluble.

Frank and Krüger (1894) found that spores of different Peronosporaceae and other fungi were killed when immersed for 24 hours in bordeaux mixture several weeks old. However, when similarly treated with the filtrate from the mixture, they were not killed, but germinated. The filtrate showed no soluble copper by chemical tests, and these authors conclude that the presence of the insoluble copper is necessary to bring about the death of the spores.

Rumm (1895) placed uredospores of *Puccinia coronata* in drops of bordeaux mixture. Those spores not in contact with the bordeaux particles, not only were not injured, but germinated. Only when the spores were in contact with the particles or immediately over them, were they killed. As a result of this test, Rumm believes that bordeaux mixture is not fungicidally active at a distance from the particles. However, Doran (1923), as well as workers in the Cornell laboratory, have found that the uredospores of grain rusts are not very sensitive to the toxic action of copper fungicides.

Aderhold (1899) obtained some interesting results while studying the poisonous action of bordeaux mixture. He found that spores of *Fusicladium pirinum* would germinate on the surface of pear leaves sprayed with bordeaux mixture, provided the spores were between the flecks of bordeaux, but that there was no germination directly over the flecks. Also, the spores would germinate in drops of rain water collected from leaves that had been sprayed. Drops of bordeaux mixture were dried down, and strips of filter paper, moistened with rain water and bearing spores of Fusicladium, were placed with one end on the dried bordeaux drops. At a distance of 0 to 4 millimeters from the bordeaux drop, no germination occurred. Progressing farther away from the bordeaux, the germination improved, and at 10 to 13 millimeters distant it was normal. Aderhold believes that the fungicidal action is in evidence only during the formation of the germ tube or during invasion itself.

Clark (1902:41) appears to have followed Swingle's suggestion with an experiment. Spores of various fungi were placed in a mixture of copper hydroxide and water in which no soluble copper could be detected beforehand. The spores were killed in a short time, and traces of copper were found in the suspension, thus "showing that they dissolve more of the $Cu(OH)_2$ than was required to kill them" (page 42 of reference cited). No controls are mentioned. Clark found further that thin-walled spores,

such as those of Rhizopus and Oedocephalum, are more quickly killed than thicker-walled ones such as those of Aspergillus and Penicillium. Clark (1902:43) concludes:

> 1. The solution of that part of the $Cu(OH)_2$ of bordeaux mixture which under orchard conditions *is of fungicidal value, is chiefly accomplished by the solvent action of the fungus spores themselves*, for they have power to dissolve sufficient copper to kill themselves.
> 2. The amount of copper necessary for the destruction of the spores of parasitic fungi is probably not more than one part of soluble metallic copper to 80,000 parts water (dew).

This work was rather severely criticized by Bedford and Pickering (1910: 108–109), who, in addition, showed that copper hydroxide is slightly soluble (0.00012 per cent soluble copper) in water exposed to the air.

Schander (1904) made tests in which bordeaux mixture was sprayed on various kinds of leaves exposed to the rains and dews, and the filtrates from these were tested for soluble copper. Schander (1904:526) concludes (translation of original): "Upon the grounds of my experiments I do not believe that enough soluble copper compounds form upon the leaf to kill the spores of fungi, but I assume with Clark that, on the whole, fungi dissolve enough copper from the precipitate of bordeaux mixture adhering to the leaf to kill themselves."

Ruhland (1903), studying fungicidal action in bordeaux mixture and other basic coppers, concludes that the rôles of atmospheric agencies and plant excretions are practically negligible, but that (translation of original) "in fact the spore excretes substances which bring the $Cu(OH)_2$ into solution. The copper dissolved by the excretion which has its origin in the metabolic activities preparatory to germination now penetrates the spores" (page 199 of reference cited). The conclusion "that the liberation of the toxic quantity of copper is brought about through the excreted metabolic products of the pre-germination activities of the spore" (page 191 of reference), is based primarily on experiments in which germination tests were made with spores of *Aspergillus niger*, *Botrytis vulgaris*, Clasterosporium, and *Cephalothecium roseum*, in (1) the control, a 0.3-per-cent glucose solution, (2) the filtrate from a 24-hours-old suspension of $Cu(OH)_2$ in a 0.3-per-cent glucose solution, (3) the filtrate from a 24-hours-old suspension of $Cu(OH)_2$ in a 0.3-per-cent glucose solution in rain water, and (4) a 0.3-per-cent glucose solution containing a suspension of $Cu(OH)_2$ and many spores. Though not definitely stated, the water in (1) and (2) presumably was not rain water. After standing thus for 48 hours, the results were, for all four fungi, 100 per cent germination in (1) and (2), and from about 50 to 100 per cent germination in (3). However, Ruhland states that in (3), 100 per cent germination would result if the spores were left for three days. None of the fungi germinated in (4). In the case of (1), soluble copper could be detected in the filtrates by testing with concentrated sulfuric acid and potassium bromide. Ap-

parently the spores had absorbed, or otherwise removed from solution, only a part of the copper that they had dissolved.

Crandall (1909:294) reports, "No evidence has been obtained in support of the suggestions of authors that solution of copper occurs through the agency of germinating spores." However, no direct experiments on which to base this conclusion are mentioned.

Barker and Gimingham undoubtedly have undertaken the most extensive investigations relative to the solvent action of fungus spores. In one series of experiments (1911), spores of *Penicillium glaucum* were placed in a 1-per-cent dextrose solution plus insoluble basic copper sulfate. After a time this showed 0.0011 per cent of soluble copper, or twice as much as the controls. Similarly, Penicillium spores were placed in a dextrose solution, and, after fair growth, were filtered. The filtrate was then added to the basic copper sulfate, and 0.0075 per cent of copper was brought into solution as compared with the control, which showed 0.0004 per cent. The authors, however, point out that the conditions were abnormal in that the solvent action may have come from dead or dying cells, and also that the culture reaction was acid. Hanging-drop cultures of conidia of *Nectria ditissima* and *Sclerotinia fructigena* were made. In general, in the presence of no-excess-lime bordeaux ($10CuO$, SO_3) or of ordinary bordeaux, there was little if any germination; while in the filtrates from such compounds, there was usually good germination and fair growth, though not so good as in the controls. But with uredospores of *Puccinia hieracea*, in the presence of no-excess-lime bordeaux there was some germination and fair growth, while in the filtrates and the controls the percentage germination was high and the growth of germ tubes vigorous. The authors state (pages 86–87 of reference cited):

> The whole of these results tend strongly to support the hypothesis that certain fungi at least are able to act on the insoluble copper compound sufficiently to poison themselves, provided that they are associated closely enough with it: for in all cases copper was practically absent from the filtrate before use, so that it was obviously the presence of the insoluble compound which was necessary to give the mixture fungicidal properties. There appear to be only two explanations to satisfy the results. Either the fungus spore in its dormant condition or in the incipient stages of germination exerts a solvent action on the copper compound: or the minute traces of that substance which can be present in a state of solution act as a cumulative poison.

In another experiment, no-excess-lime bordeaux was placed inside a diffusion tube, and the filtrate on the outside. Nectria spores were sown in each, and germinated well on the outside. A few germinated well on the inside where they were not in contact with the copper particles, which had, however, largely settled. Again, no-excess-lime bordeaux was dried on glass cover slips and suspensions of Nectria spores in distilled water were added so that they overlapped the bordeaux films. The water was allowed to evaporate and the cover slips were then placed in a moist

atmosphere. The protoplasm of the spores on the film of bordeaux became badly shrunken, while the spores beyond the zone of bordeaux were apparently normal and many had germinated. From these results Barker and Gimingham conclude (pages 88 89 of reference cited):

> It seems reasonable to postulate, therefore, a solvent action on the part of the fungus which is not powerful enough normally to produce serious toxic effect except when direct contact occurs. At the same time it is evident that such action is purely local and cannot result in the bringing into solution of sufficient copper to exercise a general fungicidal action over the whole surface of a leaf.

Pickering (1912) has criticized in some detail Barker and Gimingham's (1911) conclusions, and points out that the majority of their experiments were with the no-excess-lime bordeaux ($10CuO, SO_3$), which is not entirely insoluble. These criticisms have been sharply refuted by Barker and Gimingham (1914 b).

The later work of Barker and Gimingham (1913, 1914 b) is concerned chiefly with seedling root-hairs, the action of which they consider comparable to that of germinating spores. The seedlings were placed in various copper-compound suspensions, chiefly no-excess-lime bordeaux. Ordinary bordeaux and copper carbonate also were used. Barker and Gimingham found that only when the root hairs were in contact with the particles of the copper compound did injury and death result. The root hairs not in contact escaped injury and grew. The injurious action was entirely local, and, as the authors state (1913:61), therefore "cannot be explained as a result of the production of soluble copper by atmospheric agencies." The authors emphasize the action of the cell wall, and finally conclude, in part (page 62 of reference cited):

> (1) Living cells with readily permeable walls of the unchanged cellulose type or its equivalent are able to produce and absorb soluble copper from insoluble compounds such as the basic sulphates.
>
> (2) The area over which a single cell can exert the solvent action is limited by the size of the cell or, perhaps, more accurately, by the quantity of the solvent diffusing from it. Groups of cells acting in conjunction may cause appreciable action over a wider area than an isolated cell singly.
>
> (3) The fate of the organism depends upon the relation between the amount of soluble copper produced and absorbed and the rate of growth of the organism.

Butler (1914:139), in view of his experiment with distilled and tap water which has already been discussed under atmospheric agencies, assumes that the sporangia of Phytophthora or of Plasmopara could not have an excretory solvent action, "for, so far as at present known, organic solvents of the copper precipitate of bordeaux mixtures are more active in alkaline than in neutral mixtures, and their action is not materially interfered with when tap-water is used in lieu of distilled water."

Lutman (1916) made some interesting studies, but did not definitely commit himself to any of the three hypotheses. He employed spore tests using spores of *Phytophthora omnivorum*, *Sclerotinia cinerea*, and *Ustilago*

levis. Bordeaux mixture dried on glass slides was exposed to the rain and dew to test the effect of exposure. Spores were then placed on the slides, and, though toxicity was evident, it was but very little more pronounced than in the unexposed controls. The supernatant water from exposed bordeaux mixture was found non-toxic. The toxicity of lime-water was investigated, and the alkalinity was found to be sufficient to inhibit the germination of the spores. The effect of distance from a bordeaux film also was studied. Spores of Phytophthora and Sclerotinia were sown on glass slides at certain distances from films of ordinary bordeaux or no-excess-lime bordeaux. It was found that the spores nearest the films, that is, within 1 millimeter, did not germinate. But as the distance from the bordeaux film was increased, so the percentage of germination and the growth of germ tubes increased. Copper wire also was found to inhibit the germination of these spores if they were present in its immediate neighborhood. Lutman is inclined to the view that the copper salts formed in bordeaux mixture are, when moistened, very slowly soluble (pages 34-35 of reference cited). He planned no experiment, however, to demonstrate this. His final conclusions are:

1. The fungus spores are killed by contact, either:
   (a) with the bordeaux film, giving off free copper; or,
   (b) with the alkaline solution derived from some of the lime particles.
2. The spores are inhibited from germination if they are in the region either of the bordeaux film or of the lime particles.

Cerasoli (1921) placed grape leaves which were, respectively, healthy, slightly affected with mildew, and withered with mildew — the last-named two being well covered with fungous growth — in distilled water, care being taken to cover the leaf blades only. After some days of exposure to the air, it was found that only the water containing healthy leaves gave a neutral reaction, the water in the other cases being more or less acid. The acidity was most pronounced with the leaves having the greatest amount of fungous growth. From this Cerasoli concludes that the solution of the copper salts is due to very weak acids or acid salts secreted by the parasite during its entry into the tissues, and that this acidity would easily react on the colloidal copper hydrates which he claims are formed in bordeaux mixture, thus giving rise to copper ions to which the fungicidal action supposedly is due.

Schmidt's work (1924) has already been discussed under atmospheric agencies. He was unable to demonstrate the secretion of solvent substances from spores of Botrytis, Aspergillus, Monilia, Fusicladium, and Trichothecium, that would dissolve the copper in copper hydroxide or bordeaux mixture.

The evidence to be derived from the literature relative to the hypothesis of fungous excretions is very conflicting. Some investigators apparently have clearly demonstrated this solvent action. Others have

been unable to find any such evidence. All the positive evidence seems unanimous in showing that the sphere of action of the spores is decidedly limited. As to the nature of the solvent excretions, if any, there appears to be no experimental information.

OTHER HYPOTHESES

A hypothesis that has received but scant mention in literature is one which might be called *cumulative action*. This applies to very difficultly soluble or even so-called insoluble fungicides. It is based on the assumption that absolute insolubility is impossible. Theoretically, all compounds in the presence of water may be expected to enter into solution to a certain extent. The actual amount in solution may be the merest trace, but it will exist in equilibrum with the insoluble part. If the solute be removed, it is assumed that more will enter into solution to maintain the equilibrum. In this manner an appreciable amount of the compound may eventually come into solution, provided always, however, that the solute is constantly removed from solution.

Swingle (1896) perhaps has this hypothesis in mind when he suggests that the protective action of bordeaux mixture is due to copper in solution which so weakens the germ tube of the fungus as to render penetration impossible. Swingle cites much of Nägeli's work (1893) on oligodynamic action, or poisoning by exceedingly small quantities of metallic substances in solution. Lutman (1916) is inclined to favorably consider Swingle's suggestion. On the other hand, Butler (1914) was unable to demonstrate any evidence of oligodynamic action.

Bell and Taber (1907) hold that minute traces of copper are in solution in bordeaux mixture, and that the concentration of this soluble copper is constant.

Barker and Gimingham (1911:86-87) state that the minute traces of copper in solution in bordeaux mixture may act as a cumulative poison. However, in a later paper (1913:59), they state that the minute traces of copper "which are present in solution" did not "act as a cumulative poison."

Pickering (1912), in his studies on the action of iron rods on the solubility of $10CuO, SO_3$, is inclined to this hypothesis. He was, as has been noted, able to render small amounts of copper soluble, and in discussing the low degree of solubility of $10CuO, SO_3$ he makes the significant statement (page 280 of reference cited):

> The greater part of the copper in it may pass into solution and be utilised within a comparatively short time, provided there is any body present, such as iron, to react with it and remove it from the solution. This will occur equally if it be removed by absorption into a fungus spore or the leaf of a plant, and the absorption in that case should be at a much greater rate than in these experiments, because the reacting surface exposed to a given amount of copper would be far greater. . . . . Thus we may get a very energetic absorption or

fungicidal action, with a substance showing a minimum of solubility, though the possibility of this action depends entirely on the substance passing into solution.

Barker and Gimingham (1914b:223), as a result of their work on the localized action of solvent excretions from fungus spores, say: "Pickering's explanation that death is due to absorption of minute traces of copper in solution in the continuous film of liquid covering the preparation absolutely fails to explain such cases."

There are a number of other hypotheses related to fungicidal action. They are not concerned directly with the solution of the copper, however, but primarily with the toxicity of copper. Since a study of the principles of toxicity is not within the scope of this paper, they are not discussed here.

Some workers, notably the Villedieus (1920a, 1920b, 1924), and G. Villedieu (1921, 1924), have maintained that bordeaux mixture owes its protective action almost entirely to its basicity. There is little experimental evidence in support of this hypothesis.

Hooker (1924), studying the fungicidal properties of colloidal copper hydrate, suggests that the action of the copper is a catalytic one promoting oxidation. This is similar to the view of Dubois (1923), that copper and its salts appear to destroy fungi by an action comparable to that of oxidases and peroxidases and not by direct toxic action. No experimental proof was offered in either case.

The view, especially held by a number of German investigators, that the copper is absorbed by the leaf and is thus present to protect the suscept from invasion, is not dealt with here. In any case the copper must presumably be in a soluble form in order to enter the leaf. We are thus still faced with the question of the solution of the copper.

EXPERIMENTAL WORK

PROCEDURE

In the studies conducted at this laboratory, various methods of approach have been used in an effort to detect the exertion of solvent action by the spores of fungi. The methods are similar, however, in that spore tests have been universally employed; that is, spores have been used directly to detect the presence of soluble toxic materials. We are here concerned with such minute quantities of the toxic substances that the usual chemical tests have been found inadequate. However, certain types of spores will give a very definite reaction to the presence of deleterious substances in extraordinarily small amounts. This is especially so in the case of copper. Furthermore, in nature it is the spore itself which in the final analysis must be the indicator of toxicity.

The general method and technic of testing by spores have been discussed in detail in the second paper of this series. The procedure there

described has been followed throughout, with the simple modification that the aqueous suspension of spores was added to the solution or suspension to be tested. The resulting spore suspension was then pipetted as drops on the slides, and the germination tests were thus made. In order to prevent undue dilution, a concentrated spore suspension usually was added to the solution or suspension before the latter was placed on the slides. Commonly one volume of spore suspension was added to four volumes of solution or suspension; thus the latter was diluted to four-fifths of its original strength.

In all cases, for a specific test the percentage germination is based on the counts from at least sixteen drops. Usually, however, this number has been doubled, and not uncommonly it is the average of three or four tests. The results are recorded as explained on page 21; that is, the percentage and the type (A, B, C, or D) of germination are recorded. The A type of germination is the most vigorous, having germ tubes of approximately 200μ in length or longer; in B type, the germ tubes are 100 to 200μ long; in C type, they are 25 to 100μ long; and in D type, they include those just produced and those up to 25μ in length.

It might be well to state here that the writer agrees with Clark (1902:29), that it is more desirable to consider the point of inhibition of germination rather than the death point, for, "if the germination of the spores be *inhibited* by a concentration of a copper salt, it was merely a question of time when they would be killed."

After experimenting with various fungi, conidia of the fungus *Sclerotinia americana* were finally selected as being the most suitable for these investigations. *Sclerotinia americana* (Wormald) Norton and Ezekiel is the common brown-rot fungus of stone fruits in North America (Ezekiel 1924, Wormald 1928a, 1928b). In culture it produces an abundance of fairly large conidia. These spores germinate readily in water and with a high percentage of germination. Standardized cultures (page 13) giving from about 97 to 98 per cent germination were used in all tests. The spores were from cultures five to twelve days old; distilled water was used for spore suspensions; concentrations of spore suspensions showed from fifteen to forty spores per microscopic field of 1575μ diameter; incubation was at a temperature of 21° to 26° C.; and spore-germination counts were taken from 24 to 36 hours after planting. In certain cases, tests with other fungus spores supplemented those made with *S. americana*.

THE TOXICITY OF COPPER

On the selection of *Sclerotinia americana* conidia for these investigations, it seemed desirable to obtain more nearly accurate information relative to the toxicity of copper toward these spores. For this purpose, dilute solutions of copper sulfate in distilled water were made up. At infinite

dilution, the ionization of this salt is presumably complete (Randall, 1916). Hence, at the dilutions used in these tests — that is, 0.1 to 10 milligrams of copper per liter of copper-sulfate solution — the copper sulfate is from 96 to practically 100 per cent ionized, or nearly all in the ionized state.

In studying the toxicity of copper-sulfate solutions toward spores, at least four factors must be considered. These are: the age of the spores; the number of spores; the concentration of copper in solution; and the volume of solution.

In table 1 is recorded the germination of spores of different ages in varying dilutions of copper-sulfate solutions. These figures are based on the results from a number of tests. The tests are in close agreement. The concentration of the spore suspensions was from approximately 30 to 32 spores per low-power field of the microscope. In all future references to concentration of the spore suspension in this paper, the number of spores is designated for a low-power field. The low-power field of the microscope employed is 1575μ in diameter. The area of the field is thus approximately 1/30 of that of the entire drop, since the drops are from 8 to 10 millimeters in diameter. Also, since the drops are 1/20 cubic centimeter in volume, the volume of solution in a low-power field would be approximately 1/600 cubic centimeter. The temperature was from 21° to 25° C.

TABLE 1. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN DILUTE COPPER-SULFATE SOLUTIONS: RELATIONS OF COPPER-SULFATE CONCENTRATION AND SPORE AGE

(Spore-suspension concentration, 30 to 32)

Age of spores, in days	Milligrams of copper per liter of solution					
	0	0.1	0.2	1.0	2.0	10.0
	Per cent and type of germination					
5	98.3 A	98.8 A	85.8 B	0.7 D	Trace	0
6	98.7 A	98.8 A	88.3 B	0.3 D	Trace	0
7	98.4 A	98.6 A	90.0 B+	0.5 D	Trace	0
8	97.8 A	98.1 A	88.2 B	0.8 D	Trace	0
10	96.6 A	96.8 A	76.9 B	0.8 D	Trace	0
12	95.3 A	92.7 A–	64.0 B–	0.3 D	0	0
17	88.5 B	79.0 B	48.3 C	0.3 D	0	0

The results, which are shown graphically in figure 1, immediately demonstrate the extreme sensitivity of *Sclerotinia americana* conidia to copper. As may be expected, the spores become less viable and more copper-sensitive with increasing age. For the younger spores, 5 to 10 days old, 0.1 milligram of copper per liter of solution is slightly stimulating. Increasing the concentration above this results in a rapid decrease in the percentage germination. Spores of the ages commonly used, and

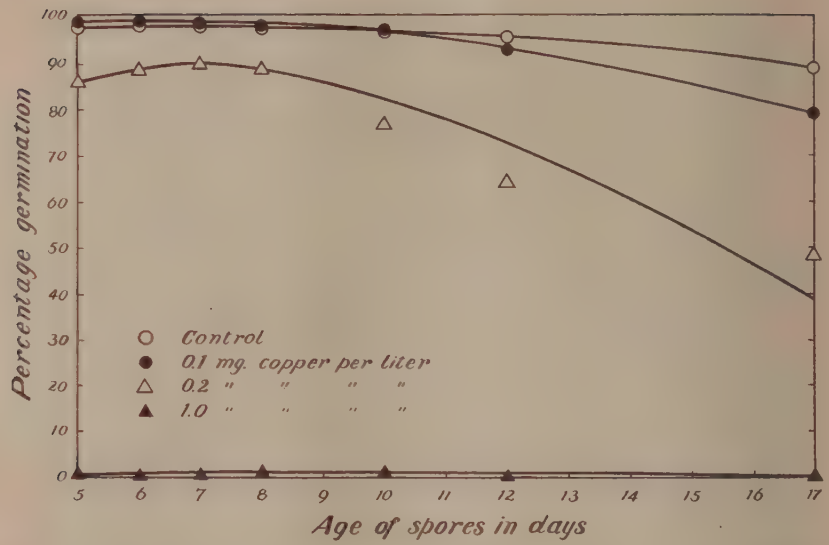

FIGURE 1. GERMINATION OF CONIDIA OF SCLEROTINIA AMERICANA IN DILUTE COPPER-SULFATE SOLUTIONS: RELATIONS OF COPPER CONCENTRATION AND AGE OF SPORES

sown at a suspension concentration of 30 to 32, are therefore definitely sensitive to as small a quantity of copper as 0.2 milligram per liter of solution. The potassium-ferrocyanide test commonly used for copper will show fairly plainly at 20 milligrams of copper per liter, and with uncertainty at 10 milligrams per liter. The Sclerotinia conidia are therefore, under these conditions, one hundred times more sensitive to copper than the potassium-ferrocyanide test.

The second step was to determine the effect of the number of spores on germination in the dilute copper solutions. The volume of solution and the spore age were held constant. Spore suspensions were obtained, of such concentrations that when they were added to the copper-sulfate solutions the resulting concentrations were as nearly as possible 4, 8, 16, and 32 spores per field. The variation from these numbers was small, being not more than 2 or 3 for the dilute concentrations, and 6 or 7 for the most concentrated. In all, five tests in duplicate were made. The ages of the spores were 7 and 8 days. Copper-sulfate solutions of from 0.1 to 10 milligrams of copper per liter of solution were employed. The results of these tests are recorded in table 2.

TABLE 2. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN DILUTE COPPER-SULFATE SOLUTIONS: RELATIONS OF COPPER-SULFATE CONCENTRATION AND NUMBER OF SPORES

(Spore ages, 7 and 8 days)

Milligrams of copper per liter of solution	Spore-suspension concentration			
	4	8	16	32
	Per cent and type of germination			
0.0	98.1 A	98.3 A	98.3 A	98.2 A
0.1	3.2 C–	58.6 B–	91.9 A–	98.8 A
0.2	Trace	4.1 C–	54.2 B–	94.5 A–
0.5	0	Trace	0.5 D	36.5 B–
1.0	0	0	Trace	0.6 D
5.0	0	0	0	Trace
10.0	0	0	0	0

These results are shown graphically in figure 2, where the percentage germination is plotted against the concentration of copper. The curves for the four concentrations of spore suspension are drawn.

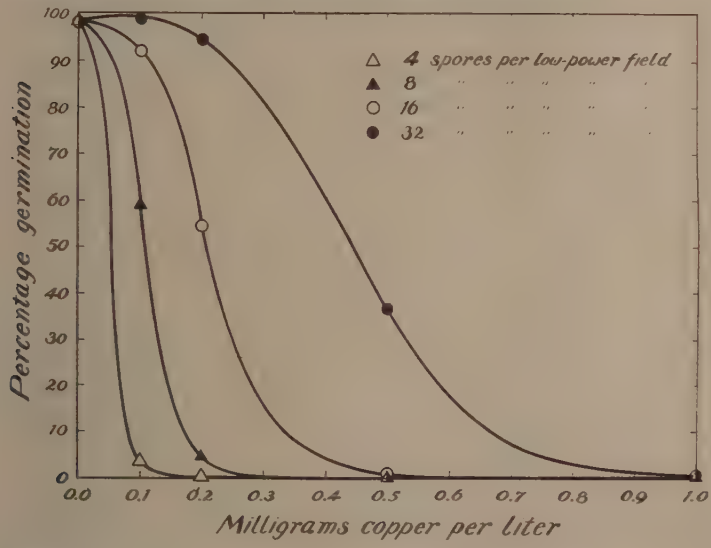

FIGURE 2. GERMINATION OF CONIDIA OF SCLEROTINIA AMERICANA IN DILUTE COPPER-SULFATE SOLUTIONS: RELATIONS OF COPPER CONCENTRATION AND NUMBER OF SPORES

In order that the relations between the number of spores and the total amount of copper may be the more clearly shown, figure 3 has been constructed from figure 2. In figure 3, milligrams of copper per liter of solution are plotted against number of spores, and four germination curves — 0, 5, 50, and 90 per cent — as derived from figure 2 are drawn.

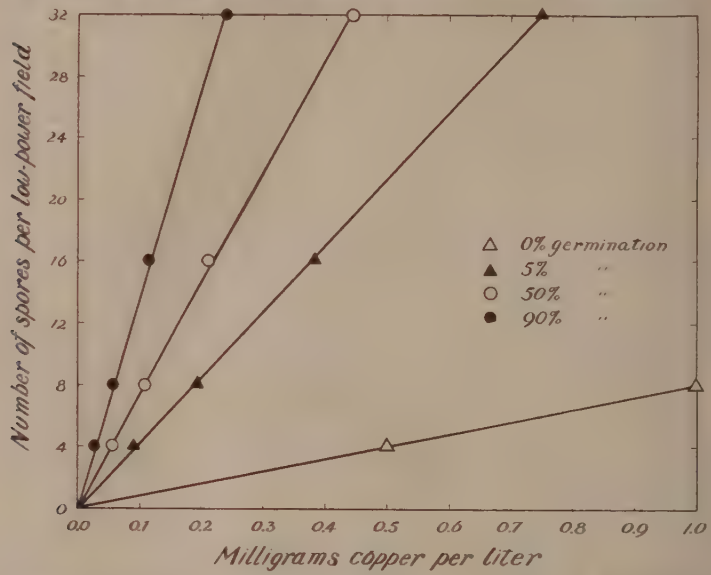

FIGURE 3. TOXICITY OF DILUTE COPPER-SULFATE SOLUTIONS TO THE CONIDIA OF SCLEROTINIA AMERICANA

Thus these are shown to be straight-line relations; that is, for a given percentage of germination the number of spores is directly proportional to the concentration of copper. This, of course, assumes that the volume of copper-sulfate solution is constant and small. In other words, there is a direct proportion between the number of spores and the total amount of copper for a given percentage germination.

In the study of the volume of solution, some difficulty was experienced. The spores will germinate well only in small volumes of water. In large volumes the oxygen relations appear unsatisfactory. A "drip system" was finally devised for a study of the larger volumes. Essentially this consists in the dripping of a large volume of solution over the spores, which are held in very shallow petri dishes. The control spores give

fairly satisfactory germination here. For the medium volumes, that is, from 1 to 25 cubic centimeters, petri dishes were employed. The smallest volumes were placed directly on slides and a drop of spore suspension was added. With the large volumes, the drops of spore suspension were first placed on the bottom of empty petri dishes and allowed to stand for about thirty minutes before the solution was added. The spores had then settled to the surface of the dish, to which they adhered, and were not readily dislodged. They were thus easily found for the purpose of making germination counts.

In this manner two sets of tests were performed. A copper-sulfate solution of a concentration of 0.1 milligram of copper per liter, was employed throughout. The volumes of solution are expressed as drops, in order that the ratio of drops of solution to one drop of spore suspension may be readily seen. The concentration of spore suspension prior to the dilution was constant and was approximately 16. The age of the spores was 7 days. The percentage of germination observed in these tests is recorded in the third column of table 3.

TABLE 3. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN DILUTE COPPER-SULFATE SOLUTIONS: FACTOR OF SOLUTION VOLUME

(Concentration of solution, 0.1 milligram of copper per liter; spore-suspension concentration, 16; spore age, 7 days)

Volume of solution: ratio of drops of spore suspension to drops of copper-sulfate solution	Total copper concentrated into one drop and expressed as milligrams per liter	Per cent of germination observed	Per cent of germination theoretically to be expected from figure 2
1:0	0.0	98.2 A	98.3
1:1	0.05	98.6 A	97.0
1:2	0.13	97.8 A	86.0
1:5	0.42	94.5 A–	3.0
1:10	0.91	81.8 B	1.0
1:20 (1 cc.)	1.9	3.9 D	0
1:50 (2.5 cc.)	4.9	Trace	0
1:100 (5 cc.)	9.9	0	0
1:200 (10 cc.)	19.9	0	0
1:500 (25 cc.)	49.9	0	0
1:1000 (50 cc.)	99.9	0	0
1:2000 (100 cc.)	199.9	0	0
1:5000 (250 cc.)	499.9	0	0
1:10000 (500 cc.)	999.9	0	0

A study of these figures shows the importance of the volume factor. A small volume of very dilute copper-sulfate solution may be entirely nontoxic toward a given number of spores, but a large volume of this same concentration is decidedly toxic to the spores. This confirms the observations noted before, that toxicity is governed by the number of spores and

the total amount of copper present, and not by the relative concentration. Similar conclusions have been drawn by Burrill (1907:568-569), Briggs (1923), and others.

In order that an adequate comparison of the volume relations may be made, the total copper present in these solutions has been calculated on the basis of milligrams of copper per liter of solution. That is, the total copper present is assumed to be concentrated into a volume of one drop. Having the concentration of copper expressed thus, by a reference to figure 2 the germination theoretically to be expected for 16 spores may be found. These germinations are given in the fourth column of table 3.

A comparison of the observed and the theoretical germination shows a great difference. Over the narrow range of volumes at which germination occurred, it will be seen that the observed germination was approximately equivalent to the theoretical for a volume only about one-fourth as great. The same total amount of copper present in a very large volume of solution is therefore less toxic than that present in a small volume.

This will appear contradictory to the previous statement, namely, that toxicity is dependent on the total copper present regardless of volume or concentration. Thus it would seem that toxicity is not entirely independent of volume, the total copper content being the same. This difference may be explained as being due to the rate of diffusion. In the case of a large volume of a dilute solution, the particles, or ions, of copper are presumably rarer in a given space than they would be in a small volume containing the same number of copper ions. Thus, in the former case, in the proximity of the spore there would be present fewer ions than in the latter case. So the final toxicity would, in a sense, be the resultant of the rate of diffusion of the copper ions and the rate of germination of the spores. The toxicity of a dilute copper-sulfate solution toward a given number of spores is therefore determined, not only by the total amount of copper present, but also to a certain extent by the rate of diffusion of the copper ions.

The results of these preliminary studies have shown that the toxicity of dilute copper-sulfate solutions to the conidia of *Sclerotinia americana* is dependent, not only on the concentration of the solution, but also on the age of the spores, the number of the spores, the total amount of copper present, and the volume of the solution. With increasing age the spores become more copper-sensitive. The toxicity, or inhibition from germination of a given number of spores, is directly related to the total amount of copper present in a given volume of solution. With the total copper content and other factors held constant, the solutions become less toxic with increasing volumes, the limiting factor being presumably the rate of diffusion of the copper.

Spores from 5 to 10 days old, present to the extent of less than 1000 to a drop of solution, are markedly sensitive to 0.2 milligram of copper per liter of solution. This is one hundred times more sensitive than the potassium-ferrocyanide test.

THE TOXICITY OF CALCIUM COMPOUNDS

Since a number of the common copper protectants contain calcium in various forms, a few preliminary tests were made to determine the relative toxicity of certain of these calcium compounds. The calcium compounds present in excess-lime bordeaux mixtures and copper-lime dust are calcium hydroxide, calcium sulfate, and calcium carbonate. Calcium hydroxide and calcium sulfate are both moderately soluble in water at 25° C., the former to the extent of 1.541 grams per liter expressed as $Ca(OH)_2$, and the latter 2.755 grams per liter expressed as $CaSO_4 \cdot 2H_2O$ (Cameron and Bell, 1906). Calcium carbonate is soluble in distilled water only to the extent of 19.6×10^{-5} gram mols per liter at 25° C. (Askew, 1924).

Four experiments were performed with calcium hydroxide, the first two in triplicate and the third and the fourth in duplicate. A saturated solution of $Ca(OH)_2$ was obtained by slaking a high grade of commercial stone lime with distilled water, making milk of lime, and filtering. From this filtrate various dilutions were made. This source of $Ca(OH)_2$ was employed in the first three experiments; for experiment 4, Baker's Chemically Pure $Ca(OH)_2$ was used. The toxicity of all was determined in the usual manner. The results are presented in table 4.

TABLE 4. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN CALCIUM-HYDROXIDE SOLUTIONS

Concentration of $Ca(OH)_2$ solution, expressed as per cent of saturation	Commercial $Ca(OH)_2$			Baker's Chemically Pure $Ca(OH)_2$
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	Per cent and type of germination			
100	0	0	0	0
50	Trace	Trace	0	Trace
25	17.3 A–	31.0 A–	4.3 A–	1.2 A–
12	96.3 A	92.1 A	93.4 A	26.5 A
6	98.4 A	98.6 A	98.6 A	97.0 A
Control	98.3 A	98.6 A	98.4 A	98.0 A
Age of spores	5 days	7 days	5 days	5 days

Similar tests were made with calcium-sulfate solutions. The calcium sulfate was obtained from three sources — Bausch and Lomb C. P., Merck precipitated, and Baker technical. The results are shown in table 5.

TABLE 5. Percentage Germination of Sclerotinia americana Conidia in Calcium-Sulfate Solutions

(Spores 5 days old)

Source of $CaSO_4$	Concentration of $CaSO_4$ solution, expressed as per cent of saturation	Experiment 1	Experiment 2
		Per cent and type of germination	
Bausch and Lomb C. P.	100	Trace	Trace
	50	60.8 C	19.9 C–
	25	94.6 A	96.7 A
	12	98.6 A	98.8 A
	6	98.7 A	98.5 A
Merck precipitated	100	11.2 C	2.1 D
	50	97.0 A	96.2 A
	25	98.8 A	98.4 A
	12	98.4 A	98.3 A
	6	98.6 A	98.5 A
Baker tec'nical	100	9.8 C	3.2 D+
	50	96.9 A	70.0 B+
	25	98.4 A	98.6 A
	12	98.6 A	98.6 A
	6	98.5 A	98.3 A
Control	...	98.4 A	98.4 A

The toxicity of calcium carbonate was tested, using the material as a dust. Three tests were made, the slides being dusted in the usual manner. On the addition of the aqueous spore suspension, a saturated solution of the calcium carbonate would presumably result and be present together with the very great excess of solid dust particles. The results are shown in table 6.

TABLE 6. Percentage Germination of Sclerotinia americana Conidia on Slides Dusted with C. P. Calcium Carbonate

	Experiment 1	Experiment 2	Experiment 3
	Per cent and type of germination		
Calcium carbonate	98.3 A	98.3 A	98.5 A
Control	98.4 A	98.4 A	98.5 A
Age of spores	9 days	9 days	9 days

These tests show that a clear saturated solution of calcium hydroxide in water is decidedly toxic toward conidia of *Sclerotinia americana*. Normal germination is obtained only when the solution has been diluted to 6 per cent of saturation.

Solutions of calcium sulfate also are toxic. They are, however, approximately only one-sixth as toxic as similar concentrations of calcium-

hydroxide solution. It is perhaps possible that the greater toxicity of the calcium-hydroxide solution is due to its alkalinity. No determination has been made of the possible toxicity of the sulfate radical or ion, either in these tests or in those with the copper sulfate.

Calcium-carbonate dust displays no toxic action toward the spores of *Sclerotinia americana.*

THE LIBERATION OF THE COPPER FROM THE PROTECTANT

In studying the manner in which the copper is rendered soluble, various types of experiments have been employed. These experiments have, in turn, been directed toward proving or disproving certain of the more important current hypotheses. In the presentation of each study, the hypothesis is first stated, and this is followed by a statement of the experiments and experimental data, and finally by a discussion of the results.

Special emphasis has been laid on the hypothesis of spore excretions. The first two of these studies have dealt with the action of atmospheric agencies. The next three relate to the rôle of spore excretions, the third of these being concerned especially with the sphere of activity of the spore. Finally, brief studies have been made of the cumulative-action hypothesis.

Atmospheric agencies

The solubility of copper protectants in distilled water

Hypothesis. Copper protectants in distilled water may be brought into solution when normally exposed to the action of the air. A copper protectant is applied to a glass surface, and in one case distilled water is allowed to remain standing on it for varying lengths of time. After exposure thus to the air, spore-germination tests are made in the filtrate. In the second case, a suspension of spores is placed directly on the fungicide and the germination is recorded. If atmospheric factors alone are operative in the solution of the copper, then in the first case there should be little or no germination, and the toxicity of the filtrate should increase with the time of standing; while in the second case, because of the short exposure to the air the germination should be good. However, if an action of the spores is the main factor, then in the first case none of the filtrates, regardless of the time of standing, should be toxic; but in the second case, because of the presence of the copper compound there should be little or no germination.

Experiments. The copper protectants employed in these experiments were bordeaux mixture (4–4–50), Pickering or no-excess-lime bordeaux (2.5–2–50), copper-lime dust, C. P. copper carbonate, and copper hydroxide. The freshly made bordeaux mixtures were tested for free copper by the use of potassium ferrocyanide, and none was found. *Sclerotinia americana* conidia were used in the germination tests.

The fungicide to be tested was sprayed or dusted into open petri dishes. Liquid fungicides were allowed to dry thoroughly. Distilled water was then added to each, about 10 cubic centimeters to a dish, and the whole was allowed to stand exposed to the air for 24 hours. The suspensions were then filtered through filter paper and the filtrates tested for free copper with potassium ferrocyanide. None was found in any test.

Coincidentally, similar but freshly made protectants were applied to glass slides. No free copper was detected here with potassium ferrocyanide. After the liquid fungicide had thoroughly dried, spore-germination tests were performed, (a) in the filtrates and (b) directly on the sprayed or dusted slides.

The average of three tests with bordeaux mixture 4–4–50, no-excess-lime bordeaux 2.5 2–50, and copper-lime dust, are given in table 7.

TABLE 7. Percentage Germination of Solerotinia americana Conidia

Treatment	Bordeaux 4–4–50	Bordeaux 2.5–2–50	Copper-lime
	Per cent and type of germination		
Filtrates	94.7 A	98.2 A	93.5 A
Treated slides	0	0	0
Control	98.5 A	99.2 A	98 5 A

Tests were likewise made with copper carbonate and copper hydroxide. The copper carbonate was obtained from four sources and all was C. P. material. The fourth sample was carefully and thoroughly washed and dried before testing. The copper hydroxide, also C. P., was obtained from two sources. Two separate tests, each in duplicate, were made on all of these materials. The spores were from 5 to 7 days old. The results are shown in table 8.

TABLE 8. Percentage Germination of Solerotinia americana Conidia

Treatment	C. P. copper carbonate				C. P. copper hydroxide	
	Samples				Samples	
	1	2	3	4	1	2
	Per cent and type of germination					
Filtrates	0	0	0	0	0	0
Treated slides	0	0	0	0	0	0
Control	98.6 A	98.6 A	98.4 A	98.4 A	98.2 A	98.6 A

Discussion. The results of the first series of experiments show clearly that practically no copper goes into solution in the distilled water which stands on the bordeaux mixtures and the copper-lime dust. It therefore

seems improbable that atmospheric factors act, at least over a period of 24 hours, through the medium of distilled water to promote the liberation of copper. The possibilities of "meteoric waters" and longer periods of standing have not, of course, been precluded here. However, it is very evident that in those cases in which the fungus spores were placed directly on the insoluble protectant, the latter was decidedly toxic; that is, in some manner the mere presence of the insoluble protectant is sufficient to inhibit the germination of the spores. This may be explained by assuming that the spores excrete a solvent capable of dissolving some of the copper.

The experiments with C. P. copper carbonate and C. P. copper hydroxide resulted contrarily to expectations. These copper compounds are ordinarily considered insoluble in water (Holland and Gilligan, 1928). Apparently this is not strictly true, for it is very evident that the filtrates contain substances in solution which are toxic to the spores. Presumably this toxic material is soluble copper. The filtrates from the copper carbonate gave no copper test when treated with potassium ferrocyanide; the copper-hydroxide filtrates, however, gave a fairly clear test. Thus the amount of copper present in the former is probably less than 10 milligrams per liter, while in the latter it is probably more nearly 20 milligrams per liter. This unsuspected fact of soluble toxic substances precludes the possible use of these copper compounds in subsequent studies of this kind.

The solubility of copper protectants in distilled and rain water, under forced aeration

Hypothesis. It is possible that in nature the copper protectants are more exposed to the action of carbon dioxide and other atmospheric agencies, than they are with plants merely standing in the laboratory. Also, rain waters perhaps possess a greater solvent action than does distilled water.

Experiments. A series of experiments were accordingly performed, in which a prolonged and enforced action of the air and of rain water was tested. Bordeaux mixture (4-4-50) and copper-lime dust were used. The test fungi were conidia of *Sclerotinia americana* and of *Macrosporium sarcinaeforme.* The latter fungus was used only in one test. It was found, however, to be approximately as copper-sensitive as *Sclerotinia americana.*

The fungicides were placed in specimen jars of 175 cubic centimeters capacity. The bordeaux mixture was sprayed on the inner side of the jars, and also on both sides of glass slides. Four of these slides were placed in each jar, this serving to increase the surface exposed. After thorough drying, about 50 cubic centimeters of distilled water was added

to each jar. The jars were then connected to a water suction pump in such a manner that fresh air was continually and vigorously drawn through the water, bathing the fungicide-coated surfaces. The water level was kept constant by daily additions of fresh distilled water. A second set was maintained, in which the fungicides were merely placed in open petri dishes and water was added as in the previous method.

In the first and second tests the air was allowed to bubble through for 9 days. In the third test this period was 8 days. At the end of these periods all the suspensions — those in the jars through which air had been passed, and those standing in the open petri dishes — were filtered through ordinary laboratory filter paper. Chemical tests with potassium ferrocyanide and potassium mercuric thiocyanate at this point failed to demonstrate the presence of soluble copper in any of the filtrates. At the outset of the experiments, similar tests of the filtrates from the fungicides had failed to show any soluble copper.

Germination tests were made with the filtrates. Although the filtrates failed to react to the chemical tests, it was thought that sufficient copper might be present to warrant a dilution. Thus, to attempt a differentiation of the filtrates, they were diluted with distilled water to one-half, one-third, and one-quarter strengths. These concentrations — full, one-half, one-third, and one-quarter strengths — are respectively designated as filtrates 1, 2, 3, and 4. The results of the germination tests are shown in table 9.

TABLE 9. Percentage Germination of Spores in Filtrates from Distilled Water on Copper Protectants Exposed to Various Treatments

Experiment no.		1		2		3	
Duration		9 days		9 days		8 days	
Fungus spores		*Macrosporium sarcinaeforme* 15 days old		*Sclerotinia americana* 4 days old		*Sclerotinia americana* 4 days old	
Treatment	Dilution	Air passed through	Standing in open dish	Air passed through	Standing in open dish	Air passed through	Standing in open dish
		Per cent and type of germination					
Bordeaux (4–4–50) filtrates	1	96.3 A	98.6 A	87.0 C	94.1 C	27.6 D	20.0 D
	2	96.6 A	96.3 A	99.1 A	98.6 A	69.3 C	63.0 C
	4	98.8 A	....	98.3 A	....	....	....
Sprayed slides from jar after aeration		0	....	0	....	0	....
Copper–lime filtrates	1	100.0 A	98.6 A	73.2 C	96.5 B	89.4 B	29.0 C
	2	98.8 A	100.0 A	96.5 B	97.9 B+	86.2 B	91.5 A
	4	98.4 A	....	96.3 B+	....	....	....
Controls		99.0 A		98.5 A		98.7 B+	

In a further series (4 and 5), the action of rain water was compared with that of distilled water, the air being bubbled through the jars for 6 and 5 days, respectively. For this purpose fresh rain water was collected. In these tests bordeaux mixture (4–4–50) was used. One bordeaux preparation was made as usual with distilled water, while in a second preparation rain water was used instead. The set-up was similar to that of the former series. There were four combinations — distilled-water bordeaux with distilled water, rain-water bordeaux with distilled water, distilled-water bordeaux with rain water, and rain-water bordeaux with rain water. A second set in open petri dishes was maintained as before. Chemical tests before and after showed no signs of free copper. The results of the germination tests with the filtrates, using conidia of *Sclerotinia americana*, are recorded in table 10.

TABLE 10. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN FILTRATES FROM RAIN WATER AND FROM DISTILLED WATER, ON BORDEAUX MIXTURES EXPOSED TO VARIOUS TREATMENTS

Experiment no.			4		5	
Duration			6 days		5 days	
Fungus: *Sclerotinia americana*			6 days old		7 days old	
Treatment			Air passed through	Standing in open dish	Air passed through	Standing in open dish
			Per cent and type of germination			
Distilled water, bathing sprayed surfaces	Bordeaux mixture made with distilled water	Filtrate 1	87.3 A–	87.6 B+	12.4 D	91.0 C
		Filtrate 3	96.6 A	96.7 A–	90.5 A–	97.3 A
		Sprayed slides	0	....	0	....
	Bordeaux mixture made with rain water	Filtrate 1	96.7 A	37.7 C	94.3 A	96.8 B
		Filtrate 3	98.0 A	98.5 A	99.0 A	98.4 A
		Sprayed slides	0	....	0	....
Rain water, bathing sprayed surfaces	Bordeaux mixture made with distilled water	Filtrate 1	0	96.4 A–	21.6 D	63.5 B+
		Filtrate 3	39.3 C	....	97.1 A	96.5 A
		Sprayed slides	0	....	0	....
	Bordeaux mixture made with rain water	Filtrate 1	0	46.5 B–	5.3 D	58.8 B–
		Filtrate 3	29.4 D	56.2 B	97.9 A	93.7 A
		Sprayed slides	0	....	0	....
Controls		Distilled water	98.5 A		98.8 A	
		Rain water	89.8 B+		95.7 A	

Discussion. The results presented in table 9 show that the continued passage of air through distilled water standing on bordeaux mixture or copper-lime does not promote the solution of copper any more than where the water is left undisturbed. The lower percentages of germination obtained in experiment 3 cannot be explained.

A study of the results given in table 10 shows that rain water does not allow as high a percentage of germination of the spores of *Sclerotinia americana* as does distilled water. No explanation of this can be given. This difference is evidenced, however, in both the experiments. The distilled water in these experiments shows a little more solvent action than did that in the previous ones. However, the difference is but small. The rain water appears to possess a greater solvent power than does the distilled water.

It should be noted that, in all cases, spores placed directly on the sprayed slides were completely inhibited from germination. These slides had been exposed in water to the action of the air for periods of from 5 to 9 days. This bears out a previous conclusion that the presence of the insoluble copper protectant is sufficient to inhibit germination, and shows that the copper is not appreciably leached out by long exposure to wetting with well-aerated water.

A second conclusion to be drawn from these experiments is that distilled water, either standing undisturbed or receiving a continuous passage of air, does not promote the solution of copper to an effective extent.

Thirdly, rain water exhibits greater solvent action than does distilled water. It is difficult to determine how much of this apparent toxicity is actually due to the solvent action of the rain water, for the germination is also slightly reduced in pure rain water. In any case, the results of the experiments show that sufficient copper is not brought into solution by this means to more than slightly inhibit the germination of the spores. This is especially significant when it is realized that here a very large amount of carbon dioxide came in contact with the insoluble copper for long periods of time. It is believed, therefore, that under natural conditions atmospheric agencies cannot bring about the liberation of copper necessary to kill or to effectively inhibit the germination of spores.

Spore excretions

The solvent action of filtrates from suspensions of germinating spores

Hypothesis. The hypothesis considered in the following paragraphs is essentially that of Wallace, Blodgett, and Hesler (1911:186), namely, "that the germinating spore secretes a dissolving agent which acts on the insoluble fungicide to bring small quantities of the same into solution"; that is, water in which spores have germinated possesses dissolving powers greater than that of pure water.

Wallace, Blodgett, and Hesler (1911) performed a few preliminary experiments with lime-sulfur. They placed spores of *Sclerotinia cinerea*[2] in water in a watch glass, allowed them to germinate for three days, and then filtered the suspension. This filtrate they considered to consist

[2] Presumably *S. americana*.

of water plus the hypothetical dissolving agent secreted by the spores. They report (page 187 of reference cited):

> Some drops of the filtrate were placed on slides sprayed with lime-sulfur 1 25 and some on clean slides. Germination tests in water in which no spores had previously germinated were carried through the entire experiment as a check. All slides were kept in moist chambers for two days, then the water from each slide was removed to clean ones, and spores added.

The germination results were as follows: control, 40 per cent; water from sprayed slide, 8 per cent; water plus spore filtrate from clean slide, 25 per cent; and water plus spore filtrate from sprayed slide, 0 per cent. A near duplicate of this experiment gave somewhat similar results.

Experiments. The method employed by the author was in principle similar to that of Wallace, Blodgett, and Hesler. In some preliminary experiments the results were not satisfactory, largely because of an apparent staling effect of the water in which the spores had germinated. The spores employed here were those of *Botrytis allii*. In all later tests, conidia of *Sclerotinia americana* were used and excellent results were obtained. This modification, together with a greater familiarity with the method, resulted in the elimination of the staling-effect factor.

The fungicides used were colloidal bordeaux membranes from 4-4-50 bordeaux mixture, and copper-lime dust. The colloidal membranes were separated from the mixture by centrifuging. The clear limewater and lime particles were thus eliminated. Neither the original bordeaux mixture nor the bordeaux membranes showed any free copper by the potassium-ferrocyanide test. The bordeaux membranes were sprayed into petri dishes and allowed to dry for 12 hours. With the copper-lime dust, the petri dishes were first sprayed with distilled water, and then the dust was applied and allowed to dry likewise.

Spores of *Sclerotinia americana* were placed in distilled water in open petri dishes and allowed to germinate for 12 hours. The germination period was thus reduced because in nature spores would not normally germinate for as long periods as two and three days before fungicidal action would become operative. In all cases the germination was uniformly about 97 per cent. These suspensions were then filtered, and the resulting solution constituted the "old spore filtrate." Ten cubic centimeters of this was added to each of a number of petri dishes, in which the bordeaux membranes or copper-lime dust had been placed previously. At the same time, ten cubic centimeters of distilled water was placed in an equal number of petri dishes, likewise coated with bordeaux membranes or copper-lime dust. The remainder of the old spore filtrate was stored in a Pyrex beaker. All preparations were allowed to stand exposed to the air for 36 hours, and then filtered.

Twelve hours previous to this filtering, a fresh spore suspension had been prepared and allowed to germinate for 12 hours. This suspension

was then ready to be filtered at the same time as those described above. It constituted the "new spore filtrate." The second spore filtrate was prepared in order that possible changes occurring in the old spore filtrate during the period of standing might be checked.

The solutions after filtering were thus from (I) old spore filtrate, (II) new spore filtrate, (III) distilled water on fungicide, and (IV) old spore filtrate on fungicide. To test these solutions for toxicity, spore-germination tests were made with conidia of *Sclerotinia americana*. However, in order to have comparative tests — that is, to balance the possible staling effects of the spore filtrates — equal volumes of the spore filtrates were added to equal volumes of solution III. This necessitated adding an equal volume of distilled water to solution IV, so that the respective dilutions would be approximately equal.

The final solutions in which the spore-germination tests were then made, consisted of (1) control, distilled water, (2) old spore filtrate, (3) new spore filtrate, (4) solution III plus old spore filtrate, (5) solution III plus new spore filtrate, and (6) solution IV plus distilled water.

The germination tests were made in the usual manner. In general, nos. 1, 2, and 3 were carried in duplicate, 4 and 5 in triplicate, and 6 in quadruplicate, moist-chamber tests. In this manner six separate experiments were made with bordeaux membranes and five with copper-lime dust. The results appear, respectively, in tables 11 and 12.

TABLE 11. Solvent Action on Bordeaux Membranes of Filtrates from Suspensions of Germinating Spores: Percentage Germination of Sclerotinia americana Conidia in the Filtrates

	Experiment no.					
	1	2	3	4	5	6
	Per cent and type of germination					
Old spore filtrate	.....	96.2 A	96.6 A	98.0 A	96.2 A	98.0 A
New spore filtrate	62.0 C	98.2 A	97.7 A	98.6 A	96.4 A	98.1 A
Filtrate from water on fungicide; plus old spore filtrate	.....	97.6 A	95.3 A	98.1 A	37.3 B	97.9 A
Filtrate from water on fungicide; plus new spore filtrate	88.0 A	98.4 A	97.6 A	76.2 A	37.8 B	98.1 A
Filtrate from spore filtrate on fungicide; plus distilled water	11.6 C	56.4 C	3.8 D	8.9 C	7.4 C	20.8 B
Control	96.5 B	98.2 A	97.7 A	98.6 A	91.2 A	97.9 A
Age of spores, in days, for:						
Old filtrate	5	6	6	6	8	8
New filtrate	7	7	7	7	9	9
Control	8	8	8	8	10	10

TABLE 12. SOLVENT ACTION ON COPPER-LIME DUST OF FILTRATES FROM SUSPENSIONS OF GERMINATING SPORES: PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN THE FILTRATES

	Experiment no.				
	1	2	3	4	5
	Per cent and type of germination				
Old spore filtrate	98.5 A	95.7 A	98.3 A	98.3 A	98.3 A
New spore filtrate	98.7 A	98.2 A	98.3 A	98.3 A	98.3 A
Filtrate from water on fungicide; plus old spore filtrate	97.8 A	95.1 A	98.0 A	98.3 A	98.4 A
Filtrate from water on fungicide; plus new spore filtrate	98.6 A	98.1 A	98.2 A	72.6 A	98.4 A
Filtrate from spore filtrate on fungicide; plus distilled water	15.9 D	35.0 C	12.2 C	2.4 D	34.0 C
Control	98.5 A	97 2 A	97 0 A	97.0 A	98.5 A
Age of spores, in days, for:					
Old filtrate	8	7	7	7	7
New filtrate	9	8	8	8	8
Control	10	9	4	4	5

Discussion. The results of these repeated experiments with bordeaux mixture and copper-lime dust lead to but one conclusion, namely, that excretions from germinating spores are active in bringing the copper into solution from the insoluble fungicide.

Although the amount of copper rendered soluble in these experiments was not sufficient to entirely inhibit the germination of the spores, it must be borne in mind that here both the solvent principle and the soluble copper were doubtless greatly diluted. In nature the reaction of single isolated spores possibly differs, in that there is perhaps a greater concentration of soluble copper about the spore.

In these experiments the "spore filtrate," or filtrate from the solution in which the spores had germinated, was not found to be toxic to the spores even after standing for two days. This, therefore, cannot be a factor in the toxicity of the solutions. Likewise, as was previously shown, the action of atmospheric agencies on these copper protectants while in contact with distilled water was not sufficient to liberate any appreciable quantity of soluble copper.

From these experiments it cannot be determined at what period of germination, if any, the production of the excreted solvents by the spore is most abundant. It is perhaps possible that a spore, even in its normal life processes before germination has begun, may excrete dissolving materials.

However, regardless of these possibilities, these experiments seem to demonstrate definitely, at least on the part of germinating spores, an excreted solvent capable of dissolving copper from "insoluble" copper protectants.

The action of spore excretions on bordeaux mixture in dialyzing sacs

Hypothesis. If bordeaux mixture containing no free copper is placed in a suitable dialyzing membrane immersed in water, it may be assumed that the colloidal bordeaux membranes will not be able to diffuse outward. The dialyzing membrane, however, should allow the outward and inward diffusions of materials in solution. A solvent action on the part of the spore excretions being assumed, then, if the fungus spores are placed in water on the outside of the dialyzing membrane, the excretions will pass through the dialyzing membrane. Here reaction will take place with the bordeaux membranes, and by reason of the solvent powers of the spore excretions some copper will be brought into solution. This soluble copper will diffuse outward and react with the fungus spores, inhibiting their germination and ultimately killing them.

Experiments. Collodion dialyzing sacs were prepared according to the method of Eggerth (1921), d'Herelle (1926), and Mulvania (1926). The porosity of these sacs varies according to the ratio of alcohol and ether; a greater degree of porosity is obtained by increasing the percentage of alcohol. The first collodion sacs prepared were of the formula 23 77 1.5; that is, 23 cubic centimeters of absolute ethyl alcohol, 77 cubic centimeters of ethyl ether, and 1.5 grams of soluble nitrated cotton. The sacs were molded on the inside of 1.5x11-centimeter test tubes.

Bordeaux mixture (4 4 50) was prepared and tested for free copper. None was found. The bordeaux was then placed inside the collodion dialyzing sacs. The mouths of the sacs were tied with string and sealed with collodion. After this the sacs were thoroughly rinsed in distilled water and then placed in open petri dishes. Fresh distilled water was added to the dishes so that the sacs were just submerged. Two sacs were placed in each dish. The dishes were then divided into two lots. To one there was added a concentrated suspension of conidia of *Sclerotinia americana*. The other remained standing without spores. After two to three days the spores of the first lot were examined for germination. At this time the bordeaux-containing sacs were removed from the second lot of dishes and a suspension of spores was added to the remaining liquid. After standing thus for another two days, these spores were in turn examined for germination. This was to test for any possible outward diffusion of copper. Controls were carried throughout, in which a suspension of the spores had been placed in dishes containing dialyzing sacs

holding distilled water only. In this manner three experiments were performed in duplicate. The germination results are given in table 13.

TABLE 13. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN WATER IN CONTACT WITH DIALYZING SACS CONTAINING BORDEAUX MIXTURE

Experiment no.	1		2		3	
Collodion solution	23–77–1.5		23–77–1.5		25–75–2.5	
	Per cent and type of germination					
Spores on outside of dialyzing sacs containing bordeaux	0	0	0	0	0	0
Solution in which dialyzing sacs of bordeaux had stood	95.5 A	98.0 A	98.3 A	98.6 A	89.0 A–	89.0 A–
Control	99.7 A		98.7 A		95.0 A	
Age of spores	4 days		8 days		12 days	

These experiments were further elaborated. An effort was made to determine the effect of dialyzing sacs of varying permeability. Sacs were prepared in three degrees of permeability — 20–80–2.5, 30–70–2.5, and 40–60–2.5 — and the experiments were run in triplicate with each type. To obviate any complications due to the presence of excess lime, 4–4–50 bordeaux was prepared and then centrifuged. The centrifuging made possible the separation of the clear limewater and lime particles from the jelly-like bordeaux membranes. Water was added to the bordeaux to bring it back to the original strength. The mixture was chemically tested for free copper, and none was found. The mixture was then placed in the collodion sacs, and these were tied and were sealed with agar. The sacs were placed in pairs in petri dishes containing distilled water, and allowed to stand for two days. The water was then pipetted off and germination tests were made in it. These results are shown in the first column of percentages in table 14. A spore suspension of *Sclerotinia americana* was then added to the water surrounding the collodion sacs in the petri dishes. The germination was observed after two days, and is recorded in the second percentage column of table 14. In order to demonstrate whether any free copper was left in solution — that is, whether more copper had been dissolved than was taken up by the spores — the liquid surrounding the sacs was filtered and germination tests were made in the filtrate. These results are given in the third percentage column of table 14. Finally, to find whether the collodion sacs had leaked, the sacs were carefully rinsed in distilled water, placed in fresh distilled water, and allowed to stand for five days. The water was then pipetted off and germination tests were made in it. The results of this final test are recorded in the last column of table 14.

TABLE 14. Percentage Germination of Sclerotinia americana Conidia in Water in Contact with Dialyzing Sacs of Varying Permeability Containing Colloidal Bordeaux Membranes

Treatment	Set no.	Water from dishes holding sacs containing bordeaux	Spores placed in water surrounding sacs of bordeaux	Filtrates from the aqueous suspension of percentage column 2	Bordeaux sacs from percentage column 2 washed, and placed in fresh water for 5 days: water from same
		Per cent and type of germination			
Collodion 20–80–2.5	1	98.8 A	92.7 A	95.1 A	96.0 A
	2	95.8 A	91.3 A	96.4 A	96.0 A
	3	97.1 A	93.4 A	92.0 A	.....
Collodion 30–70–2.5	4	96.2 A	86.1 B+	94.4 A	95.7 A
	5	97.0 A	88.3 B+	91.8 A	.....
	6	97.0 A	89.4 A–	97.1 A	95.6 A
Collodion 40–60–2.5	7	85.5 A	31.4 C	97.0 A	96.6 A
	8	95.4 A	39.4 C	95.7 A	86.0 A
	9	93.4 A	38.6 C	93.4 A–	.....
Control	..	95.2 A	97.5 A	97.4 A	96.6 A
Age of spores.....	..	10 days	10 days	5 days	9 days

Discussion. The results of the first three experiments appear to clearly substantiate the hypothesis of a solvent action on the part of the spore execretions toward the insoluble copper compound.

The results of the fourth experiment do not demonstrate this so clearly. The figures in percentage columns 1 and 4 of table 14 show that the collodion sacs were entire and did not leak. Hence, when they were standing in pure water there was no appreciable outward diffusion of copper. This would imply that no copper comes into solution merely on standing. The results recorded in percentage column 3 show that all of the copper which may have been brought into solution was absorbed, adsorbed, or otherwise removed from solution by the spores, or possibly also by the process of filtering.

The figures in percentage column 2 show that collodion sacs of 20–80–2.5 formula are practically impervious to the inward diffusion of any solvent, or the outward diffusion of copper, or both. The collodion sacs of 30–70–2.5 formula permitted a slight outward diffusion of copper, while the most permeable sacs, 40–60–2.5, permitted a considerable outward diffusion of copper. By a comparison with the three former experiments (table 13), it would be expected that with the collodion sacs of greater porosity — that is, 30–70–2.5 and 40–60–2.5 — sufficient copper would be allowed to diffuse outward to completely inhibit germination of the spores. It is beyond the scope of this paper to attempt to explain this difference in

diffusion. However, it does appear possible that the difference in the alkalinity of the two types of bordeaux may have been a contributing factor. So far as is known, all other factors were the same.

The results of this series of experiments are believed to point definitely to a solvent action exercised by some excretion from the fungus spores.

The sphere of influence of spore excretions

Hypothesis. It is assumed that actual contact of the spore with the colloidal particles is not necessary in order that the copper be rendered soluble.

In the preceding study there was definite indication that this is the case, and an effort has accordingly been made to determine how great the separation between the spore and the copper particle may be before a lessening of toxicity is evident; in other words, to determine the sphere of activity of the solvent spore excretions, and hence of the liberated copper particles. It will be recalled that Lutman (1916) observed that no spores germinated when within a radius of one millimeter from a drop of Pickering's bordeaux. On the other hand, Barker and Gimingham (1911, 1913, 1914 b) have maintained that actual contact, or at least very close association, is necessary for toxic action.

Experiments. In a preliminary experiment, bordeaux mixture 4-4-50 was prepared which showed no free copper by the potassium-ferrocyanide test. The bordeaux was sprayed into four watch glasses and allowed to dry for 24 hours. Ten cubic centimeters of distilled water was then added to each glass, and the whole was allowed to stand thus exposed for 60 hours. In the meantime, fresh bordeaux mixture containing no free copper was placed in the form of drops on clean glass slides. Two drops were placed on each slide, each drop about 1.5 centimeters from the end of the slide. These drops were allowed to dry down for 24 hours. The end of this period coincided with the end of the 60-hours period referred to above.

A spore suspension of *Sclerotinia americana* conidia was prepared and two drops were placed on each of a number of clean glass slides. Two drops were placed also on each of the slides holding the dried drops or areas of bordeaux mixture. The drops of spore suspension and of bordeaux were separated by a space of from 1 to 5 millimeters.

The spores were allowed to settle, and thus to adhere to the surface of the slides. This required about half an hour. Then, to the slides with the bordeaux and spore-suspension areas, distilled water was added by means of a pipette, to form a film covering and connecting each pair of areas — bordeaux and spores. Thus, although the bordeaux and the spores were connected by a bridge of water, there was no direct contact between them.

The water standing in the watch glasses sprayed with bordeaux was filtered, and the filtrate was added to the clean slides holding the spore-suspension drops, so that it formed a covering film. This film resembled that of the distilled water on the other set of slides. This set constituted a check on the copper that might come into solution from the bordeaux through atmospheric agencies or other means.

A control was maintained, in which films of distilled water were placed over drops of spore suspension. All slides were placed in moist chambers and the usual germination tests were made. The usual type of control also was carried.

The germination results after 24 hours are recorded in table 15.

TABLE 15. Sphere of Influence of Spore Excretions: Percentage Germination of Sclerotinia americana Conidia

Treatment	Per cent and type of germination
Bordeaux filtrate on spores	58.2 B
Bordeaux areas near spore areas, distilled water over all	0
Water-film control	96.5 A
Control, usual (spores 6 days old)	97.8 A

The reduced germination in the case of the filtrate is doubtless due in large part to soluble calcium compounds. The ungerminated spores here appeared normal, which is characteristic of spores killed by $Ca(OH)_2$ and $CaSO_4$. However, the spores that were placed near the areas of bordeaux mixture showed a very evident precipitation of their contents, a condition characteristic of copper poisoning.

This experiment was elaborated in some detail. Drops of bordeaux mixture (no free copper) were placed, one each, on a number of slides and allowed to dry down. Then to one slide was added a drop of *Sclerotinia americana* spore suspension, and an additional second drop twice as far from the edge of the bordeaux as the first drop. On a second slide there were placed three drops of the spore suspension, on a third slide four drops, and on a fourth slide five drops. A slide with five drops is diagrammatically illustrated in figure 4. This series was made up in quadruplicate. The spores were allowed time in which to settle and adhere to the glass. The average distance between the edge of the bordeaux area and the edge of the nearest spore-suspension drop was 7.5 millimeters. Drop no. 2 was 15 millimeters distant, no. 3 was 30 millimeters, no. 4 was 40 millimeters, and no. 5 was 50 millimeters. Regardless of the number of spore-suspension drops on each slide, these distances were maintained. The number of spore-suspension drops was varied in order to further study the correlation between total toxic substance and number of spores, which has already been discussed in the copper-toxicity studies.

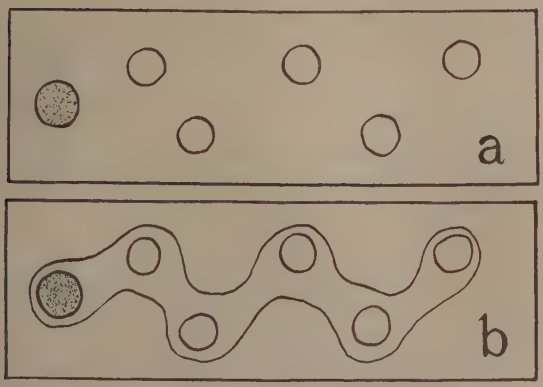

FIGURE 4. SPHERE OF INFLUENCE OF SPORE EXCRETIONS

a, A slide with a dried drop of bordeaux mixture and five drops of spore suspension. b, The same, with the drops connected by a film or bridge of water

To increase the distance and the number of spores, petri dishes were employed. A drop of bordeaux mixture was placed near the edge of a dish. On an arc of a circle 2 centimeters distant, three drops of spore suspension were pipetted, each drop as far away from the others as possible. In addition, three drops were placed on three other arcs, which were respectively 4, 6, and 8 centimeters distant from the bordeaux area. The spores were given time to adhere to the glass. This series also was carried in quadruplicate.

The drops on the slides were connected by a film of water, as was done in the preliminary experiment. With the petri dishes, the whole was flooded with a thin film of water. Germination tests were made in the usual manner, and the average results for each series are recorded respectively in tables 16 and 17. A control similar to that in the preliminary

TABLE 16. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA AT VARYING DISTANCES FROM AN AREA OF DRIED BORDEAUX MIXTURE (SPORE-SUSPENSION DROPS AND BORDEAUX DROP PLACED ON SLIDES AND CONNECTED BY A BRIDGE OF WATER)

	Distance of spore-suspension drops from bordeaux area				
	7.5 mm.	15 mm.	30 mm.	40 mm.	50 mm.
	Per cent and type of germination				
2 spore-suspension drops per slide	0	0	.....	.....	.....
3 spore-suspension drops per slide	0	13.8 D	36.6 C+	.....	.....
4 spore-suspension drops per slide	0	15.3 D	34.5 C+	53.0 B	.....
5 spore-suspension drops per slide	0	15.4 D	41.0 B–	61.6 B+	71.8 A
Bordeaux filtrate	66.2 A				
Control	94.6 A				

TABLE 17. Percentage Germination of Sclerotinia americana Conidia at Varying Distances from an Area of Dried Bordeaux Mixture (Spore-Suspension Drops and Bordeaux Drop Placed in a Petri Dish and the Whole Covered by a Film of Water)

Distance of spores from bordeaux area				Control
2 cm.	4 cm.	6 cm.	8 cm.	
Per cent and type of germination				
79.6 C	87.8 B–	89.9 B+	90.0 A–	94.1 A

experiment was carried, in which the filtrate from water standing on a surface sprayed with bordeaux mixture was tested. The age of the spores was 6 days.

Discussion. The results from the tests of the spores placed on the slides at varying distances from the bordeaux mixture, are very interesting. It can be seen that as the distance from the bordeaux and the number of spores are increased, so the percentage and the quality of germination increase. With the petri dishes, the volume of water and the relative distances are sufficiently great so that the differences in percentage and quality of germination are not so pronounced.

Undoubtedly the filtrate from water standing in bordeaux mixture is slightly toxic. However, as has already been pointed out, it is believed that this toxicity is due to certain soluble calcium compounds present, and perhaps also to the alkalinity of the filtrates. A point that seems to substantiate this is the apparent normal appearance of the contents of ungerminated spores, which is characteristic of inhibition by calcium. Spores killed or inhibited from germination by copper show a very decided precipitation of their contents.

All of these experimental results demonstrate most clearly the fact that direct contact is not necessary in the toxic action of bordeaux mixture on spores. It is evident that the spores may be killed, or at least inhibited from germination, while at some considerable distance from the particles of bordeaux. A factor influencing this toxicity is the number of spores present. It is possible, either that the solvent spore excretions can operate over a wide area, acting on bordeaux particles to liberate copper by which the spores themselves are ultimately killed, or that the cumulative toxic action may account for their death. Or, again, both conditions may bring about this result. It seems difficult to conceive of such minute traces of copper — as required by the cumulative-action hypothesis — diffusing at such a rate as to inhibit the germination of the distant spores.

Cumulative action

The abstraction, by spores, of copper from large volumes of water flowing over copper protectants

Hypothesis. The protective action in copper fungicides, especially bordeaux mixture, may be dependent on a cumulative action of the copper in equilibrium in solution. That is, bordeaux mixture may contain always in solution a minute trace of copper infinitely beyond detection by ordinary chemical tests or even spore tests. In the presence of spores or other reactive bodies, this soluble copper is absorbed or otherwise removed from solution. An equilibrium having existed between the insoluble bordeaux and the soluble copper, this is now disturbed. Therefore, more copper will enter into solution to maintain the equilibrium. The spores will in turn remove this copper, and so on until finally the spores will have accumulated sufficient copper to ultimately destroy themselves. This, of course, assumes that the source of soluble copper, the bordeaux particles, is always present.

Experiments. In a study of this hypothesis, some preliminary experiments were performed. It was thought that if this hypothesis were true, then large quantities of water slowly dripped over "insoluble" copper protectants and then over the spores not in contact with the fungicides would carry traces of soluble copper to the spores. Because of the constantly changing equilibrium conditions about the fungicide, fresh copper would continually enter into solution and the spores would abstract enough copper to eventually become poisoned. If, however, the excretions of spores are the only solvent agent, then the spores would not be killed. So if the water were first dripped over spores capable of excreting the solvent agent, and then the water plus this agent were passed over the fungicide, sufficient copper should be brought into solution to kill the test spores.

An apparatus was accordingly devised and set up. Tests were made with spores of *Sclerotinia americana* and bordeaux mixture 4–4–50, Woburn bordeaux, and copper-lime dust. Since the experiment was, in general, unsatisfactory, a description of the apparatus is considered unnecessary. The chief difficulties encountered were the poor germination of the spores in the control apparatus, the rapid disorganization of the Woburn bordeaux, precipitating black copper oxide — which occurred also, though to a less extent, in the bordeaux mixture and the copper-lime — and the precipitation of calcium compounds from the bordeaux mixture and the copper-lime.

The results of three typical experiments are given in table 18.

TABLE 18. Percentage Germination of Sclerotinia americana Conidia in Flowing Water Which Had Previously Been Dripped over Copper Protectants

	Experiment no.		
	2	4	5
Fungicide	Woburn bordeaux	Bordeaux 4–4–50	Copper–lime
	Per cent and type of germination		
Apparatus control	55.8 B–	90.7 C	78.7 B–
Water dripped over germinating spores	41.2 B–	70.9 C–	61.6 B–
Water dripped over fungicide	0	0	17.2 D
Water dripped over germinating spores, then over fungicide	0	0	0
Control (usual)	97.5 A	97.6 A	97.8 A

Discussion. No conclusions can be drawn from these experiments because of the instability of the fungicides, which introduced new and unknown toxic factors. With the copper-lime dust there is slight evidence of a solvent action on the part of the germinating spores.

The rate of solution of soluble and "insoluble" copper compounds

Hypothesis. If suspensions of soluble and "insoluble" copper compounds are prepared in various dilutions in a parallel series, the soluble copper compounds should be immediately toxic to the extent of their total copper content. On the other hand, according to the cumulative-action hypothesis only a very small trace of the "insoluble" copper compound is at any one time in solution. It is only after a period of time that an appreciable amount of the copper can be brought into solution, and this is dependent on some reactive body being present to remove it as fast as it comes into solution. Presumably, complete solution and removal of all the copper from the "insoluble" compound would require considerable, if not almost infinite, time. Therefore, with two dilutions of the same total copper content, that of the soluble copper compound should be very much more toxic than that of the "insoluble" compound, for with the latter a period of time must elapse before any appreciable percentage of the total copper can have come into solution. If, however, spore-excretion solvents are active, there should be little difference in toxicity, though probably the soluble compound would be somewhat more toxic because of its immediate action.

Experiments. Dilute solutions of copper sulfate were prepared. Suspensions of an "insoluble" proprietary copper dust, of equal total copper content, were likewise prepared. Spores of *Sclerotinia americana* were added to these solutions and suspensions, and germination tests were made. The recorded germinations are shown in table 19.

TABLE 19. PERCENTAGE GERMINATION OF SCLEROTINIA AMERICANA CONIDIA IN SOLUTIONS OF COPPER SULFATE AND SUSPENSIONS OF AN "INSOLUBLE" COPPER DUST

Milligrams of copper per liter of solution	Experiment 1		Experiment 2	
	Soluble $CuSO_4$	"Insoluble" Cu dust	Soluble $CuSO_4$	"Insoluble" Cu dust
	Per cent and type of germination			
20.0	0	0	0	0
10.0	0	0	0	0
2.0	Trace	0.6 D+	Trace	0.6 D
1.0	2.2 D	14.6 D+	1.5 D	17.3 C–
0.2	86.2 A–	96.5 A–	80.7 B	87.5 B+
0.1	97.4 A	97.6 A	96.0 A	96.8 A
Control	97.7 A		95.5 B+	
Age of spores	5 days		12 days	

A similar experiment was performed with copper sulfate and insoluble copper phenyl acetylide (CuC_8H_5).[3] Spores of *Macrosporium sarcinaeforme* were used. The results appear in table 20.

TABLE 20. PERCENTAGE GERMINATION OF MACROSPORIUM SARCINAEFORME SPORES IN SOLUTIONS OF COPPER SULFATE AND SUSPENSIONS OF INSOLUBLE COPPER PHENYL ACETYLIDE

Milligrams of copper per liter of solution	Soluble $CuSO_4$	Insoluble CuC_8H_5
	Per cent of germination	
10,000.0	0	0
1,000.0	0	0
100.0	0	91.6
10.0	Trace	97.0
1.0	2.8	97.2
0.1	96.3	97.1
Control	97.0	97.0

Discussion. Following the reasoning advanced above, the results shown in table 19 seem to point clearly to a solvent action on the part of the fungus spores. However, it is possible also that any cumulative action may be masked by that of the spore-excretion solvent.

In table 20, spore-solvent action appears to be much less marked. This perhaps may not be unexpected, since copper phenyl acetylide is not ordinarily used as a copper protectant and hence need not necessarily be expected to possess protectant properties. On the other hand, there is a lack of evidence in support of the cumulative-action hypothesis.

In conclusion it may be stated that these preliminary experiments fail to give any very substantial evidence on the cumulative-action hypothesis.

[3] The copper phenyl acetylide was kindly furnished by Professor J. R. Johnson, of the Department of Chemistry, Cornell University.

THE NATURE OF THE SPORE EXCRETIONS

In the foregoing studies, experimental evidence seems to establish the presence of an excretion from the spores which exerts a solvent action on the insoluble copper protectant, thus freeing soluble copper. It is hoped that, at some future date, investigations regarding the nature of the spore excretions may be undertaken. At this time only some preliminary studies will be mentioned.

None of the investigators who have been inclined to the spore-excretion hypothesis appear to have attempted a study of the nature of the spore excretions. In the case of germinating seeds and the roots of young plants, Barker and Gimingham (1914 a) state that carbon dioxide is an active agent. However, they submit no experimental evidence. Other workers also have suggested the possible rôle of carbon dioxide.

Attempts have been made to determine the hydrogen-ion concentration of the water in which spores were germinating. Spores of *Sclerotinia americana* were allowed to germinate in distilled water for varying periods of time. Hydrogen-ion determinations were attempted of suspensions of germinated and ungerminated spores and of filtrates from each. Two standard methods were used — that of the quinhydrone electrode, and that of indicators. The results were very unsatisfactory. No constant readings could be obtained, nor could any consistent difference between these solutions and distilled-water controls be demonstrated. Apparently these solutions are almost, if not entirely, devoid of buffer action, and accurate determinations of the hydrogen-ion concentration are impracticable if not impossible. One interesting observation was made, however, namely, that in the process of filtration the filtrate is rendered more alkaline, usually to the extent of pH 0.3 to 0.5 more than the original solution.

Following the failure to obtain satisfactory results in the hydrogen-ion determinations, some preliminary tests were made in determining total acidity by titrations. To insure a very sensitive test, the method of Rosa, Vinal, and McDaniel (1913, 1914), employing iod-eosine as the indicator, was used.[4] Five-cubic-centimeter samples of the spore-excretion water were taken, and were titrated with N/1000 NaOH. Hydrogen-ion determinations of samples of distilled water which had been exposed for the same length of time that the spores were allowed to germinate — from 16 to 28 hours — also were made. The solutions were not filtered.

The distilled-water controls required from 13/20 cubic centimeter of N/1000 NaOH through neutrality, to 4/20 cubic centimeter of N/1000 HCl, for neutralization to iodeosine. The spore-excretion solutions were found to be more acid than the water, and a fairly constant difference of about 5/20 cubic centimeter of N/1000 NaOH was observed. That is, the spore excretions had an acidity equivalent to N/20,000 HCl.

[4] The writer wishes to acknowledge his indebtedness to Dr. A. S. McDaniel for assistance and advice in performing these tests.

Titrations were made also with the indicator brom thymol blue. The difference obtained in this case was from 4/20 to 7/20 cubic centimeter of N/1000 NaOH. The two indicators thus agree well with each other.

The actual acidity of the spore excretions of *Sclerotinia americana* conidia from these preliminary tests appears to be very slight. It is possible that some weak organic acid or acids are excreted by the fungus spores, and that this condition accounts for the solvent action.

SUMMARY

1. An extensive review of the literature relative to the liberation of copper from insoluble copper protectants is presented in this paper. In the main, three hypotheses have been advanced by investigators to explain this: (1) that of atmospheric agencies; (2) that of suscept excretions; and (3) that of the dissolving action of spore excretions. A less common hypothesis is that of the cumulative action of the toxic agent in equilibrium solution. The present study is concerned primarily with investigating the hypothesis of the dissolving action of spore excretions. Some attention has been given also to the hypothesis of cumulative action.

2. The experimental procedure employed was that of testing by spore germinations, as described in the second paper of the series. In general, conidia of *Sclerotinia americana* were used.

3. The toxicity of copper-sulfate solutions toward conidia of *Sclerotinia americana* was found to be positively correlated with total copper content and negatively correlated with number of spores. Total copper content must be calculated on the basis of concentration and volume of solution. However, with increasing volumes, other factors being constant, the solutions become relatively less toxic, presumably because of the factor of diffusion. With increasing spore age, given solutions become more toxic. *Sclerotinia americana* spores 5 to 10 days old, present to the extent of about 1000 per 0.05 cubic centimeter of solution, are markedly sensitive to 0.2 milligram of copper per liter of copper-sulfate solution.

4. The toxicity of certain calcium compounds toward conidia of *Sclerotinia americana* was studied. Chemically pure calcium carbonate was found to be non-toxic, but C. P. calcium-hydroxide and C. P. calcium-sulfate solutions were somewhat toxic. Calcium-hydroxide solutions ceased to be toxic at dilutions of 0.1 milligram per liter of solution, while calcium-sulfate solutions ceased to be toxic at 0.7 milligram per liter.

5. The filtrates from C. P. copper carbonate and copper hydroxide in distilled water contained quantities of soluble copper sufficient to kill the spores of *Sclerotinia americana*.

6. The action of atmospheric agencies was investigated. In no case was copper brought into solution in toxic concentrations in distilled water which had stood on glass surfaces coated with bordeaux mixtures or copper-

lime dust. When air was forcibly drawn through the distilled water which had stood on the bordeaux mixture (dried) or the copper-lime dust for periods of 5 to 9 days, the resulting filtrates were rendered only slightly toxic. If rain water, which exhibits greater solvent action, was substituted, the filtrates were more toxic. However, rain water in itself does not allow quite as good germination of the spores of *Sclerotina americana* as does distilled water. The action of atmospheric agencies in rendering copper soluble from insoluble protectants seems to be relatively unimportant.

7. The filtrate from water in which spores of *Sclerotinia americana* had germinated, when placed on bordeaux mixture (dried) or copper-lime dust for 2 days, invariably rendered the filtrate from the resulting suspension toxic. The filtrate from suspensions of germinating spores was non-toxic. The control solutions — that is, the filtrates from pure water which had stood on the protectant — always gave a high percentage of germination. This seems to demonstrate the excretion of a solvent agent by the germinating spores.

8. Bordeaux mixture placed in collodion sacs of a certain degree of porosity, and the whole placed in distilled water, did not appear to give a diffusible soluble copper, since the outside water was non-toxic to the spores when the sacs were removed. If, however, spores were placed in the water in the presence of the bordeaux-containing sacs, they did not germinate, thus indicating the production by the spores of some solvent agent capable of diffusing through the dialyzing membrane to free diffusible copper.

9. In all the experiments performed, the copper protectants were definitely toxic only in those cases in which the spores, or filtrates from suspensions of germinating spores, were able to maintain an aqueous connection with the protectant. Spores placed at varying distances from a drop of dried bordeaux mixture, and connected therewith by a bridge or film of water, were entirely inhibited from germinating at a distance of 7.5 millimeters. As the distance of the spore from the bordeaux increased, the percentage germination of the spores also increased. This clearly demonstrates that direct contact is not essential for toxic action by bordeaux mixture or copper-lime dust.

10. No definite evidence could be obtained, either in favor of or against the cumulative-action hypothesis — that is, the accumulation of copper, by the spores, from the very minutes traces presumably present in solution in equilibrium with the " insoluble " copper protectant.

11. Preliminary tests seemed to indicate that the spore excretions of *Sclerotinia americana* were very slightly acid, to the extent of about the equivalent of N/20,000 HCl.

12. All the evidence obtained in this investigation appears to demonstrate that the chief agency in bringing about the liberation of copper

from insoluble copper protectants is spore excretions. Various atmospheric factors, especially meteoric waters, may be supplementary agents, and perhaps also the excretions from suscept parts, though this has not been clearly established. In any case, these agents appear to be nonessential for protective action. In addition, various calcium compounds in the case of bordeaux mixture and copper-lime dust, and probably also the alkalinity of the mixture, play a rôle. Finally, there may be an infinitely slight accumulation of copper from that which presumably is always present in equilibrium with the most "insoluble" of copper protectants. No definite conclusions can be drawn from the preliminary experiments performed to study this point.

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